UNIVERSIDADE DE PASSO FUNDO
FACULDADE DE AGRONOMIA E MEDICINA VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM AGRONOMIA

OCCURRENCE AND APPLICABILITY OF ARBUSCULAR MYCORRHIZAL FUNGI IN STRAWBERRY

OCORRÊNCIA E APLICABILIDADE DE FUNGOS MICORRÍZICOS ARBUSCULARES EM MORANGUEIRO

ANA CLÁUDIA PEDERSEN

Thesis submitted to the Programa de Pós-Graduação em Agronomia of Faculdade de Agronomia e Medicina Veterinária of UPF in partial fulfillment of the requirements for the degree of Doctor in Agronomy.

Passo Fundo, April 2016

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Programa de Pós-Graduação em Agronomia
Faculdade de Agronomia e Medicina Veterinária - FAMV

A Comissão Examinadora, abaixo assinada, aprova a tese

"OCORRÊNCIA E APLICABILIDADE DE FUNGOS MICORRÍZICOS ARBUSCULARES EM MORANGUEIRO"

Elaborada por

Ana Cláudia Pedersen

Como requisito parcial para a obtenção do grau de
"Doutora em Agronomia – Área de Produção Vegetal"

Aprovada em: 02/05/2016
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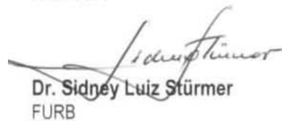

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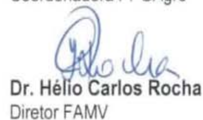

Dr. Hélio Carlos Rocha
Diretor FAMV

CIP – Catalogação na Publicação

P371o Pedersen, Ana Cláudia

Occurrence and applicability of arbuscular mycorrhizal fungi in strawberry / Ana Cláudia Pedersen. – 2016.

187 f. : il.; 25 cm.

Orientadora: Profa. Dra. Eunice Oliveira Calvete.

Tese (Doutorado em Agronomia) – Universidade de Passo Fundo, 2016.

1. Morango - Cultivo. 2. Fungos – Aplicação na horticultura. 3. Solo – Uso. I. Calvete, Eunice Oliveira, orientadora. II. Título.

CDU: 634.75

Catalogação: Bibliotecária Cristina Troller - CRB 10/1430

"*The role of arbuscular mycorrhizal fungi may be critical if agriculture is to return to the state where luxury levels of farm inputs of fertilisers, pesticides and or chemicals are decreased to levels that are still economic, yet do not pollute the environment or pose health risks to consumers or handlers*."

Bethlenfalvay & Lindermann (1992)

To my parents, I dedicate.

ACKNOWLEDGMENTS

To my family who has always given me love and strength.

To Dra. Eunice O. Calvete, for her guidance and support throughout the course of this research.

To Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) for the grant.

To the committee member for all suggestions and teachings throughout the doctorate.

To Dr. Sidney L. Stürmer for your help throughout the doctorate; for providing his laboratory (FURB) to perform some evaluations; the donation of mycorrhiza species to perform the experiments.

To Dr. Pedro M. Antunes for guidance during in Canada. And also, I would like to thank his family, Paula, Vera and Matias for making my time in Canada a wonderful experience.

To farmers, both in Brazil and in Canada, for making available their areas to sample soil and plants.

To Dra. Becky Hughes for providing the micropropagated plants for the experiment in Canada.

The students Renata, Marcos, Cassiano, Nicolas, José Luís, Damiana, for all the help and friendship. And also, Fabiola and Rosiani for their guidance and friendship.

My best friend, Giovani, for always being by my side in stressful times.

And everyone who contributed directly or indirectly to accomplish this work.

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OCCURRENCE AND APPLICABILITY OF ARBUSCULAR MYCORRHIZAL FUNGI IN STRAWBERRY

ANA CLÁUDIA PEDERSEN[1]

ABSTRACT - Most of the strawberry crop systems use high quantities of inputs. This management practice has become an important factor in determining the profitability of crops, because the cultivars selected in breeding programs have a high potential for productivity and quality in response to these management practices. Strawberry has a high response to fertilizers, pesticides, irrigation, and other management practices. A strategy able to contemplate these aspects is the use of beneficial soil microorganisms as arbuscular mycorrhiza (AM) fungi, able to colonize plant roots and thus establish mutualistic association with plants. The use of AM fungi in horticulture is important, particularly, due to the ability of these fungi to increase the absorption of nutrients, resistance to diseases and environmental stresses. In order to know the effects of these microorganisms the present study aimed to assess whether there is diversity of mycorrhizal fungi in soil under strawberry cultivation and inoculation with these fungi affects the growth in different stages of development, production and fruit quality. Five experiments were carried out with the following objectives: 1) survey the occurrence of AM fungal species present in different sites under strawberry crop; (2) test if AM fungi inoculation in strawberry plants affect leaf

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[1] Biologist, doctorate student of Programa de Pós-Graduação em Agronomia da Faculdade de Agronomia e Medicina Veterinária of Universidade de Passo Fundo.

appearance rate and phyllochron during development stages; (3) evaluates the effect of AM fungi inoculation on growth, development and yield of strawberry growing under soilless system conditions; (4) test if inoculation with AM fungi could increase quality parameters of strawberry fruits growing under soilless system conditions; (5) test AM fungi inoculation of micropropagated strawberry plantlets in relation to its performance on the field. The first four experiments were carried out at University of Passo Fundo, Brazil, and fifth at Algoma University, Canada. Arbuscular mycorrhizal fungi diversity, root colonization and inoculum potential in the rhizosphere of strawberry crops show differences among the sites surveyed. The two most frequent species in soils under strawberry crop are *Claroideoglomus etunicatum* and *Funneliformis mosseae*. Inoculation with arbuscular mycorrhizal fungi in strawberry plants influences the leaf appearance rate and phyllochron during the development stages. However, did not modify biomass production, weight and diameter of strawberry fruit and yield. In contrast, the introduction of AM fungi into strawberry plants, during transplant in soilless growth system, increases the fruit quality parameters such as content of total phenolic, anthocyanins and flavonols and also the antioxidant activity. On the other hand, inoculation during acclimatization of strawberry plantlets does not influence plant establishment, growth and runner formation in the field. However, a lot still needs to be elucidated in relation to this fungus and how we can explore these microorganisms in order to maximize production and quality and minimize environmental impacts.

Key words: diversity, *Fragaria* x *ananassa* Duch., growth, quality, runner, yield.

OCORRÊNCIA E APLICABILIDADE DE FUNGOS MICORRÍZICOS ARBUSCULARES EM MORANGUEIRO

RESUMO - A grande maioria dos sistemas de cultivo de morangueiro utiliza elevada quantidade de insumos. A utilização dessa prática de manejo tornou-se um fator importante na determinação da rentabilidade das culturas. Isto porque, as cultivares selecionadas em programas de melhoramento apresentam um elevado potencial de produtividade e qualidade em resposta a essas práticas de manejo. Essas cultivares tem uma alta resposta aos fertilizantes sintéticos, agroquímicos, irrigação, entre outras práticas de manejo. Uma ferramenta capaz de melhorar esses aspectos no cultivo do morangueiro é o emprego de microrganismos benéficos do solo. Dentre esses microrganismos constam os fungos micorrízicos arbusculares (MA) capazes de colonizar as raízes e, assim, estabelecer associação mutualísticas com as plantas. A utilização de fungos MA na horticultura está ligado, principalmente, a habilidade desses fungos em aumentar a absorção de fósforo e outros nutrientes, na resistência à doenças e à estresses ambientais. Com o objetivo de conhecer a ação destes microorganismos, o trabalho avaliou a ocorrência de fungos MA em solo sob cultivo do morangueiro e se a inoculação com esses fungos interfere no crescimento nos distintos estádios de desenvolvimento, produção e qualidade dos frutos. Foram realizados cinco experimentos com os seguintes objetivos: 1) avaliar a ocorrência

de espécies de fungos MA em diferentes locais sob cultivo do morangueiro; 2) testar se a inoculação com fungos MA afeta o aparecimento de folhas e o filocrono em diferentes estádios fenológicos do morangueiro; 3) avaliar se há efeito da inoculação micorrízica no crescimento, desenvolvimento e na produtividade do morangueiro sob sistema de cultivo sem solo; 4) testar se a inoculação com fungos MA aumenta a qualidade de frutos de morangueiro cultivado em substrato e 5) testar se a inoculação com fungos micorrízicos arbusculares, em muda micropropagadas de morangueiro, em relação ao seu desempenho à campo. Os quatro primeiros experimentos foram desenvolvidos na Universidade de Passo Fundo, Brasil, e o quinto na Universidade de Algoma, Canadá. A diversidade de fungos MA, colonização micorrízica e o potencial inóculo na rizosfera da cultura do morangueiro apresenta diferenças entre os locais avaliados. As duas espécies mais frequentes nesses solos cultivado com morangueiro são *Claroideoglomus etunicatum* e *Funneliformis mosseae*. A inoculação com fungos MA no transplante de mudas de morangueiro, no sistema de cultivo sem solo, influencia a taxa de aparecimento de folhas e o filocrono durante as fases de desenvolvimento. Todavia, não altera a produção de biomassa, massa e tamanho dos frutos e produtividade. Em contraste, há aumento nos parâmetros de qualidade dos frutos como conteúdo de fenólicos totais, antocianinas e flavonóides, assim como na ativdade antioxidante. Por outro lado, a inoculação com fungos MA durante a aclimatização de mudas micropropagadas de morangueiro não influencia positivamento o estabelecimento, crescimento e formação de estolões à campo. Contudo, muito ainda precisa ser elucidado em relação a esses fungos

e como podemos explorar os microrganismos do solo a fim de maximizar a produção e minimizar impactos ambientais.

Palavras-chave: diversidade, *Fragaria* x *ananassa* Duch., crescimento, qualidade, estolão, produtividade.

1 INTRODUCTION

Strawberry is cultivated worldwide with high profitability. It is one of the most consumption fruit because its flavor and, in recent years, has attracted attention for its nutritional value.

Crop management practices that aim greater fruit yield and quality with lower environment impact is required. A strategy able to contemplate these aspects is the use of beneficial soil microorganisms as arbuscular mycorrhiza (AM) fungi, able to colonize plant roots and thus establish mutualistic association with plants.

The effects of symbiosis on the growth and development of horticulture crops has been studied with different goals and divergent responses. The interest in this biotechnology is due to the benefits such as enhancing nutrient and water uptake and protection against biotic and abiotic stresses.

The benefits of AM fungi in strawberry culture are known, but little is known about the occurrence of these fungi in soil under this crop. Also, little is known about the effects of these microorganisms during development stages of strawberry produced in substrate or during plantlet stage.

Strawberry in soilless crop system is characterized by high use of agricultural inputs. The contribution of these microorganisms in the nutrient uptake, plant growth, resistance to pathogens and stress are well established. However, much remains to be elucidated about the potential of this practice and how we can explore the use of these

microorganisms in order to maximize yield and minimize environmental impacts.

Inoculation with AM fungi presents divergent responses regarding yield, quality and plant resistance with positive, none and negative reports. These differences are due mainly to the interaction between plant species (and cultivar) and fungus species. Thus, the inoculum choice becomes a critical step to obtain the benefits that symbiosis can promote. In addition, growth conditions, such as soil properties and climate, influence the responses.

Thus, the following questions are made: Which species of AM fungi occur in soil under strawberry crop? What are the effects of AM fungi in different stages of development of strawberry plants grown in substrate? What are the benefits of inoculation with AM fungi in the yield and fruit quality of strawberry plants grown in substrate? What are the benefits of inoculation in strawberry plantlets?

The following main objective was set to answer those questions: evaluate the occurrence of AM fungi in soil under strawberry crop and whether inoculation with these fungi interfere growth in different stages of development, yield and fruit quality.

It was proposed the following specific objectives:

· Survey the occurrence of AM fungal species present in soil under strawberry (cv. Camarosa) crop systems;

· Evaluate the effect on the type of mycorrhizal inoculum on strawberry growth, development and yield growth under soilless system during crop season;

· Test if inoculation with AM could increase quality parameters of strawberry fruits growing under soilless system conditions;

· Determined whether AM fungal pre-inoculation of strawberry micropropagated plants during the acclimatization stage benefit plant growth and clonal plant production in the field.

2 REVIEW

2.1 Strawberry

2.1.1 History

The cultivated strawberry (*Fragaria* × *ananassa* Duch.) originated approximately 250 years ago (DARROW, 1966). The first historical reports of the strawberry crop were recorded in the 14th century, when some wild species of *Fragaria* were grown in European gardens with medicinal and ornamental purpose (DARROW, 1966). In 1624, plants of wild strawberry species, *F. virginiana* were taken to France, as well as in 1714 plants of *F. chiloensis* were also taken to Europe. By the 18th century the situation of the culture began to change in the gardens of the Palace of Versailles due to natural cross between these two species. *F.* x *ananassa* originated from a natural hybridization between two octoploids *F. virginiana* and *F. chiloensis* (DUCHESNE, 1766; DARROW, 1966).

Currently the botanical classification accepted for commercial cultivars is *Fragaria* x *ananassa*, produced and appreciated worldwide. From there, the strawberry crop has spread throughout for most of Europe and the Americas.

2.1.2 Economic importance

The cultivated strawberry belongs to the Rosaceae family, is a globally consumed horticulture crop that is grown worldwide. The importance of this crop takes place by the high profitability compared to other crops, widely acceptance by consumers and the industry (FACHINELLO et al., 2011). Among berry fruits, strawberry crop

covers producers with different production scales for different markets (SPECHT & BLUME, 2009). Global strawberry production is twice the amount of all other berry crops combined (STEWART, 2011). In addition, it is one of the most common fruits of frequent human consumption because, besides its attractive color and taste (MANDAIL et al., 2009), it is also have potential health benefits such as nutrients and vitamin C content (GIAMPIERI et al., 2012). They are also rich in phenolic compounds (PINELI et al., 2012), including anthocyanins, hydrolysable tannins, and phenolic acids (GIAMPIERI et al., 2012). Due to the presence of antioxidants compounds strawberry consumption in human health and disease prevention is an active research area (ROMANDINI et al., 2013).

Due to the high productivity and attractive taste this species has greatest economic importance among small fruits (OLIVEIRA et al., 2005a). It is a crop of great economic and social importance in several countries, the five top producers countries in 2013 were China (38%), United States (17.5%), Mexico (4.9%), Turkey (4.8%) and Spain (4%) (FAOSTAT). The strawberry is grown throughout the world and cultivation and production increase each year. From 2010 to 2013 world production increased 17.4%, and the highest yield was obtained in the United States, 58 t ha^{-1}, followed by Mexico, 45 t ha^{-1} (FAOSTAT).

In Latin America, Chile is the biggest producer, followed by Brazil (OLIVEIRA & SCIVITTARO, 2009). In Brazil, the culture has grown in recent years due mainly to the inclusion of more productive cultivars, crop systems and more appropriate management practices. This crop plays an important source of income for families

of small and medium farmers with approximately 0.5 to 1 ha (PAGOT & HOFFMANN, 2003). It is estimated that 90% of the national strawberry production is focused on the fresh fruit market and the remaining is destined for industry (ANTUNES & REISSER JÚNIOR, 2007).

Strawberry crops are an important socio-economic activity in many regions of Brazil. National production is around 100 thousands tones in an estimated area at 3,500 ha (CARVALHO, 2006; ANTUNES & REISSER JÚNIOR, 2007), mostly from soil cultivation (CARVALHO, 2006). This yield tends to increase as the incorporation of new technologies and cultivars (OTTO et al., 2009).

Despite not being among the main world producers Brazil begins to stand out because of the environmental conditions for strawberry crops and production in almost all months of the year (ANTUNES & REISSER JÚNIOR, 2007). National production is concentrated in temperate and subtropical regions such as Minas Gerais (41.4%), São Paulo (15.4%) and Rio Grande do Sul (25.6%) (CAMARGO FILHO & CAMARGO, 2009).

2.2 Arbuscular mycorrhizal fungi

Microbial activity in the rhizosphere is a major factor that determines the availability of nutrients to plants and has a significant influence on plant health and productivity (JEFFRIES et al., 2003). Among the components of the soil microbial community, arbuscular mycorrhizal (AM) fungi are particularly important because they are widely distributed in most ecosystems (SMITH & READ, 2008) and formed mutualistic associations between the roots of most terrestrial

plants species (SCHÜβLER et al., 2001; SMITH & READ, 2008). AM fungi are the most common microorganisms in terrestrial ecosystems (SIQUEIRA et al., 2002). Colonizing over 80% of plant species, take up the most diverse ecosystems (SIQUEIRA et al., 2002; MOREIRA & SIQUEIRA, 2006; PASZT et al., 2011; BASLAM & GOICOCHEA, 2011), and also in cropping systems, especially if managed with sustainable practices (GIANINAZZI & SCHÜEPP, 1994).

These fungi are biotrophs that require the host plant to complete their cycle, in other words fungi are fully dependent on plants for carbohydrates while their wide-spreading extraradical mycelium, in turn, provides the host nutrients and water from the soil (SMITH & READ, 2008). These symbioses promote plant growth by enhancing nutrient uptake and protection against biotic and abiotic stresses (SMITH & READ, 2008). In addition, AM fungi have an indirect influence on plant growth because of their effects on soil structure stabilization (BETHLENFALVAY & SCHÜEPP, 1994).

Given the importance of the AM fungi, there is a great interest in establishing the possible relationships between its occurrence and inoculation on agricultural productivity. Many beneficial effects on plant growth are already known. However, these effects do not depend exclusively on the fungus, but the characteristics of the plant and environmental conditions such as soil and climate.

2.3 Strawberry and arbuscular mycorrhizal fungi

The interest in symbiosis of plants with AM fungi has increased continuously during the last decades. Especially the aspects

concerning their effects on plant growth and yield. The possibilities of applying AM fungi to horticultural crops have received intense attention, especially fruit crops. Clearly, the interest of horticulturists in AM technology is due to, especially, increase in uptake of nutrients and resistance to stresses.

Crop management practices that aim greater fruit yield and quality with lower environment impact is required. A strategy able to contemplate these three aspects is the use of beneficial soil microorganisms as AM fungi, able to colonized the roots and establish a symbiosis with most of vascular plants (SMITH & READ, 2008). AM fungi structures were detected in roots of strawberry at the beginning of 20th century (WHITE, 1929). Strawberry is highly responsive to colonization with AM fungi and the benefits of this symbiosis are known demonstrating the viability of its use.

2.3.1 Plant propagation

Plant propagation in horticulture systems usually starts from seedling, cuttings, grafting or tissue culture-derived plantlets. The use of plantlets of high physiological and sanitary quality is a critical step in the strawberry crop production. The asexual reproduction through stolon, or runners, is used for commercially plantlets production, and it is a high-value cash crop. Strawberry nursery plants are characterized by their rapid propagation from mother plants to form runners and daughter plants in a short time (STEWART et al., 2005; LI et al, 2010).

For strawberry propagation, disease-free plantlets are obtained *in vitro*, although these plants start out disease free; they are

not immune. After an acclimatizing period in a substrate previously sterilized, plants are grown in nurseries for further vegetative multiplication through aboveground runners (SANTOS & MEDEIROS, 2003). The new plants (daughter plants) are rooted directly in the soil and soil-borne pests and diseases can affect plantlet quality. To reduced or eliminate this problems strawberry plants have been grown in fumigated soils (i.e. with methyl bromide).

However, these treatments controls weeds, nematodes, and soil-borne pathogens (WILHELM & PAULUS, 1980; YUEN et al., 1991) also eliminated beneficial microorganisms, such as AM fungi. Absence of beneficial microorganisms provokes negative acclimatization process and poor physiological adaptation to natural conditions (BORKOWSKA, 2002). Due to its risk to the environment some countries banned the use of this chemical (BATCHELOR, 2002), as in Brazil, what brings a major challenge for the strawberry crop worldwide, especially in plantlets production, increasing demand for alternatives. This way, AM technology by reintroducing selected AM fungi species or communities during plant propagation is a sustainable practice.

Field inoculation of crop plants is impractical due to the technical difficulties and the large amount of inoculum required (WILLIANS et al., 1992). Vegetables and small fruits producers have more efficient option using mycorrhizal technologies during plantlets production prior outplanting in order to improve survival rate and growth (RAI, 2001). Inoculation of nursery plants may be an alternative for improving plant growth and quality (GIANINAZZI et al., 1989). *In vitro* propagated crops, such as strawberry, required

much less AM inoculum than field crops and can be easily inoculated in the greenhouse, at the acclimatization stage of production, before transplanting into the filed for multiplication. After transplanting, the precolonized plants would establish a mycorrhizal hyphal network in the soil, which could serve as an inoculum source for the daughter plants (STEWART et al., 2005).

Proper use of mycorrhiza on plantlet production is beneficial (AZCÓN-AGUILAR & BAREA, 1997), as symbiosis is established before transplantation in soil (ORTAS, 2008) what can bring more possibilities of obtaining benefits from AM inoculation (CHÁVEZ & FERRERA-CERRATO, 1990). The AM inoculum can be mixed to the substrate, where plants coming from *in vitro* micropropagation, are rooted during acclimated stage (NIEMI & VESTBERG, 1992; WILLIAMS et al., 1992; VESTBERG et al., 1994; RAI, 2001; STEWART et al., 2005).

Micropropagated plants inoculated during acclimatization can obtain benefits from AM inoculation. Studies have shown that AM inoculation of micropropagated strawberry plants have benefited vegetative growth, where mycorrhizal plants produced more runners plants than the control (HRSELOVA et al., 1990; CHÁVEZ & FERRERA-CERRATO, 1990; WILLIANS et al., 1992; de SILVA et al., 1996; BOTHAM et al., 2009). But these responses can differ among the AM fungi species (NIEMI & VESTBERG, 1992; VESTBERG, 1992a; MURPHY et al., 2000a; ALARCÓN et al., 2001; TAYLOR & HARRIER, 2001), strawberry cultivar (KHANIZADEH et al., 1995; WILLIANS et al., 1992; MARK & CASSELLS, 1996), combination of cultivars and AM species

(STEWART et al., 2005) and benefits can (de SILVA et al., 1996) or cannot persist overwintering when the plants were kept in the field (VESTBERG, 1992a).

Not only more runners is possible to obtain with AM fungi but also earlier runners production (ALARCÓN et al. 2001). This way it is possible avoid problems related to delay, or also could starts strawberry production earlier (UEMATSU, 1996) when the price in the market is higher (DURNER et al., 2002; DUVAL et al., 2004).

Some works showed negative responses on growth of mother plants, such as decrease shoot dry weight, but at the same time that strongly increase runner plants production, because symbiosis affected the shift of biomass in favour of runners (HRSELOVA et al., 1990; NIEMI & VESTBERG, 1992). Contrary, KIERNAN et al. (1984) mycorrhiza affected positively the growth and also the multiplication coefficient. A negative response was also obtained in mycorrhizal plants, related to runner formation, when they grown in control conditions, otherwise positive results were obtained in the field (VARMA & SCHÜEPP, 1994).

Another alternative is inoculate micropropagated plants during *in vitro* conditions (rooting stage), because can significantly improves shoot and root growth by decreasing the osmotic potential (ELMESKAOUI et al., 1995; CASSELLS et al., 1996).

High fertilizer amounts are considered unfavourable for AM fungi formation (BIERMANN & LINDERMAN, 1983). It is possible to reduce fertilizer inputs during plantlets production by introducing beneficial microbial populations (SHARMA & ADHOLEYA, 2004) into the substrate during acclimatization stage.

Fertilizer inputs can be reduced in mycorrhizal plants to levels considerably lower than those used in commercial practice and yet plant development can be maintained (WILLIANS et al., 1992). Strawberry has an extremely high P demand during reproductive stage, which could be met by AM fungi inoculation (DUNNE & FITTER, 1989).

Inoculation of micropropagated plants with AM fungi produced more runners, but also can protect plants against pathogens after transplanting in the soil (TAUBE-BAAB & BALTRUSCHAT, 1996; BOROWICZ, 2009), enhances photosynthesis rate (BORKOWSKA, 2002; FAN et al., 2008), increase water content of the roots (HERNÁDEZ-SEBASTIÀ et al., 1999) and improves survival (SBRANA et al., 1994).

2.3.2 Nutrition

Among the factors contributing to the growth and productivity of strawberry, nutrition is the most important (UMAR et al., 2008), which includes the use of inorganic, organic and microbial sources that ensure balanced level of nutrients (LATA et al., 2013). Nevertheless the intensive use of fertilizers, especially chemical, negatively affects the quality of soil, which can result in reduced productivity and crop quality (MACIT et al., 2007).

Much of the phosphorus (P) applied to the soil through synthetic fertilizers is rapidly fixed into insoluble forms such as Ca, Fe, and Al salts, which contributes essentially to their unavailability to plants (SANYAL & DeDATTA, 1991; SINGH & KAPOOR, 1999).

Therefore, the interest in using different sources of nutrition for plants such as microbial sources.

The use of AM fungi enhances the growth of strawberry plants, as well as the nutrients uptake. Studies show that vegetative growth of strawberry was mainly due to the AM inoculation rather than level of P (HOLEVAS, 1966; KHANIZADEH et al., 1995). However, it is also observed for combinations between different cultivars and AM species (ROBERTSON et al., 1988; CHÁVEZ & FERRERA-CERRALO, 1990; WILLIAMS et al., 1992; NIEMI & VESTBERG, 1992; VARMA & SCHÜEPP, 1994), what also can result in negative responses (GRYNDLER et al., 2002; HERNÁNDEZ–SEBASTIÀ et al., 1999; de SILVA et al., 1996).

Mycorrhizal strawberry plants absorbed more P and had higher dry biomass production than non-mycorrhizal plants under phosphorus-deficient soil (HOLEVAS, 1966) and also under standard cultural conditions (DUNNE & FITTER, 1989). Hughes et al. (1978) observed the same and also higher uptake of nitrogen. Some of the responses of mycorrhizal plants are the same obtained in plants that only received fertilization (PARASKEVOPOULOU-PAROUSSI et al., 1997). In addition, other nutrients uptake has been reported in strawberry plants inoculated such as iron (Fe) and boron (B) (PARASKEVOPOULOU-PAROUSSI et al., 1997) and manganese (Mn) and magnesium (Mg) (TAYLOR & HARRIER, 2001).

Conventional agriculture practices often include abundant fertilization, aiming high productivity, leading to nutrient accumulation in the soil (STEWART et al., 2005). The establishment of the mycorrhizal proved beneficial to plants in soil with low P

available (HOLEVAS, 1966; NIEMI & VESTBERG, 1992; PARASKEVOPOULOU-PAROUSSI et al., 1997). Negative responses also were reported even for P application and AM inoculation (CEKIC & YILMAZ, 2011).

AM fungi may indirectly stimulate bacterial populations (BIANCIOTTO & BONFANTE, 2002), which can enhance phosphatase activity, P availability and uptake (GRYNDLER et al., 2002). Dual inoculation, AM fungi and plant growth promoting bacteria, can increase plant growth and nutrients uptake (GRYNDLER et al., 2002; LOWE et al., 2012).

The increased uptake of nutrients is considered a primary benefit of AM fungi (SMITH & READ, 2008) and the effect of this fungus on plant growth can be suppressed by applying nutrients (CLARK & ZETO, 2000). In other words, AM inoculation can help to reduce P fertilizer inputs (SHARMA & ADHOLEYA, 2004).

2.3.3 Yield

AM inoculation of strawberry plants might produce differing responses in terms of fruit production. Previous work on the response of strawberry to inoculation with AM fungi produced varied results, with positive responses on yield (BORKOWSKA, 2002; SHARMA & ADHOLEYA, 2004; CHÁVEZ & FERRERA-CERRATO, 1990; ROBERTSON et al., 1988; VARMA & SCHÜEPP, 1994; SINGH et al., 2010), on different crop systems (KOOMEN et al., 1987; CEKIC & YILMAZ, 2011), different inoculum (DOUDS et al., 2008) and co-inoculation with PGPB (VOSÁTKA et al., 1992; BONA et al., 2015). Contrary, no response

in yield following the inoculation was also reported (NIEMI & VESTBERG, 1992; KHANIZADEH et al., 1995; BULL et al., 2005; GARLAND et al., 2011).

The AM treatments tended to exceed during the later part of the harvesting season (CHÁVEZ & FERRERA-CERRATO, 1990), increase number of fruits per plant (KOOMEN et al., 1987; VESTBERG et al., 2000) and also enhance the size of the fruits (FRAC et al., 2009; RIVERA-CHÁVEZ et al., 2012). Fruit weight increment might be due to plant hormone produced through AM inoculation (PALENCIA et al., 2015) and cell division increase during flower development and the early stages of fruit development as a result of the greater vegetative growth (CEKIC & YILMAZ, 2011). However, AM fungi produces gibberellic acid (JAÉN et al., 1997) and cytokines which, acting in synergy, favor blooming and a higher number of fruits (VALSILAKAKIS et al., 1979), but not fruit size (RENDALEN, 1981).

2.3.4 Fruit quality

Strawberry is cultivated around the world and is appreciated, not only because of its characteristic aroma, bright red color, juicy texture and sweetness but also has been shown to be rich source vitamin C, E, β-carotene (OSZMIAŃSKI & WOJDYŁO, 2009). These berries are also rich in bioactive phenolic compounds including flavonoids, phenolic acids and anthocyanins (TULIPANI et al., 2008; OSZMIAŃSKI & WOJDYŁO, 2009; WANG & MILLNER, 2009), compounds with high antioxidant activity (HALVORSEN et al., 2002; SUN et al., 2002; MEYERS et al., 2003).

The chemical composition of strawberry varies with genotype (WANG & ZHENG, 2001; WANG et al., 2002; CORDENUNSI et al., 2002; OLSSON et al., 2004; ANTTONEN et al., 2006; CAPOCASA et al., 2008), but is also affected by factors such as agricultural practice, environment and maturity (PINELI et al., 2011; TULIPANI et al., 2011).

Studies have shown that AM fungi may be a potential strategy of biofertilization to increase not only yield but also compounds that represent the quality and nutraceutical content that can make plants more resistant (SHARMA & ADHOLEYA, 2004; MATSUBARA et al., 2009). In fruit crops, AM fungi inoculation, in addition to all benefits describe so far, also induces early flowering and fruiting (LU & KOIDE, 1994; BONA et al., 2015), affects plant hormone biosynthesis and plant metabolism (BASLAM et al., 2013) and improve the quality of crop products (CASTELLLANOS-MORALES et al., 2010).

The relationship between AM fungi colonization and secondary metabolism compounds produced in plants remains poorly understood. This symbiosis regulates genes involved in both primary and secondary metabolism of mycorrhizal plants (SALVIOLI et al., 2012).

Improvement of the content of those chemical compounds was reported in plants inoculated with AM fungi (GANZ et al., 2002; TOUSSAINT et al., 2007; BASLAM et al., 2011a,b; SCAGEL & LEE, 2012). Studies were done showing increase in strawberry fruit quality due to AM inoculation (CASTELLANOS-MORALES et al., 2010; SINGH et al., 2010; CEKIC & YILMAZ, 2011; LINGUA et al.,

2013; RAIOLA et al., 2015; BONA et al., 2015; PALENCIA et al., 2015).

Studies showed better taste in strawberry fruits, due to sugar content, when plants were inoculated with AM fungi in a soilless growing system (CEKIC & YILMAZ, 2011). The same response also was achieved in a multi-inoculation with AM fungi and plant growth promoting bacteria in conditions of low fertilizer levels (BONA et al., 2015; SINGH et al., 2010). These authors also showed healthier fruits due to higher content of ascorbic acid. Chemical compounds such as phenolics and anthocyanins also can be increased by inoculation with AM fungi (CASTELLANOS-MORALES et al., 2010; LINGUA et al., 2013), as well as antioxidant activity (RAIOLA et al., 2015).

As some of the positives results regarding AM fungi inoculation were obtained under conditions of reduced fertilization that open possibilities of increasing strawberry fruit quality in terms of sustainable agriculture, reducing chemical inputs, leading to economical, ecological and human health benefits (GIANINAZZI et al., 2010; BONA et al., 2015).

2.3.5 Plant protection - biotic and abiotic stresses

Plants are often exposed to a variety of stresses, namely biotic or abiotic, at different stages of development.

Among the biotic stresses are the pest and disease. Although the occurrence of pests in strawberry culture, such as aphids and spider mites, cause damage to production, their losses are small compared to the losses due to diseases, especially pathogenic fungi.

The incidence of disease can appear at different stages of the cycle, attacking from recently transplanted plantlets to the fruits, being an important limiting factor in strawberries production.

The main strawberry crop diseases are anthracnose (*Colletotrichum* ssp.), leafspot (*Mycosphaerella fragaria*), gray mold (*Botrytis cinerea*), leaf blight (*Dendrophoma obscurans*), verticillium wilt (*Verticillium* spp), fusarium wilt (*Fusarium* spp), angular leaf spot (*Xanthomonas fragarie*), leak (*Rhyzopus stolonifer*), powdery mildew (*Sphaerotheca macularis*), scorch (*Diplocarpon earliana*), red stele (*Phytophthora fragaria*), black root rot (*Rhizoctonia* spp., *Fusarium* sp., *Sclerotium rolfsii*, *Pythium* spp. (FORTES, 2005; REIS & COSTA, 2011).

The high incidence of diseases requires frequently application of fungicides. Furthermore, in many strawberry production fields worldwide these pathogens are controlled by soil fumigation using methyl bromide. The use of this chemical has negative consequences for the environment and increases crop costs; but also, reduces the acceptance by the consumer, which is decreasing due to lack of confidence in relation to pesticide residues in fruit (ANDRIOLO et al., 2002). Thus, alternative solutions, such as the use of microorganisms, are necessary to reduce the use of chemical and ensure the safety and productivity of strawberry fruits.

The uptake of nutrients is the focus of several studies with AM fungi. But it is clear that this association plays an important role in the suppression of pests and diseases, especially soil-borne fungal diseases (BOROWICZ, 2001; FILION et al., 2003). Several mechanisms have been proposed to explain how is the protection of

plants inoculated with AM fungi (AZCÓN-AGUILAR & BAREA, 1996; WHIPPS, 2004; DALPE, 2005; POZO & AZCÓN-AGUILAR, 2007). There is evidence that plants that absorb greater amounts of nutrients through symbiosis are more tolerant to pathogenic infections (KARAGIANNIDIS et al. 2002), but there are some cases that it does not occur (SHAUL et al., 1999; FRITZ et al., 2006).

Another factor that may help protect plants through mycorrhizal symbiosis is the number of MA fungi species in the soil. The higher number of AM fungi species larger is the protection for the host plants compare to a single species (MAHERALI & KLIRONOMOS, 2007) and also the number of pathogenic microorganisms is lower (BØDKER et al., 2002; FILION et al., 2003).

The strawberry plants responses to inoculation with AM fungi have been studied especially regarding the biological control of *Phytophthora fragaria* (ROBERTSON et al., 1988; NORMAN & HOOKER, 2000; MURPHY et al., 2000b) with some no positive biocontrol effect (BÅÅTH & HAYMAN, 1984). In some cases these effects on disease differ according to the interaction between cultivar and the AM fungi species (MARK & CASSELLS, 1996; NORMAN et al., 1996).

Reduced disease severity caused by *R. solani* (BAYÖZEN & YILDIZ, 2009) and *Fusarium oxysporum* was also reported in strawberry plants inoculated with AM fungi (MATSUBARA et al., 2004).

Although AM fungi often have been found to increase plant resistance against soil-borne diseases, AM inoculation did not

decrease the severity of crown rot (*P. cactorum*) in strawberry (VESTBERG et al., 1994). However, anthracnose (*Colletotrichum* sp.) symptoms were reduced by pre-inoculation with AM fungi, by capillary watering method (LI et al., 2006).

Arbuscular mycorrhizal fungal colonisation has also been shown to influence plant protection against insect herbivores (BENNETT et al., 2006; VANNETTE & HUNTER, 2011). By contrast, mycorrhizal strawberry plants do not exhibit higher tolerance to herbivory than non-mycorrhizal plants when challenged with the spittlebug pest *Philaenus spumarius* (BOROWICZ, 2009).

Abiotic stresses such as drought, salinity, extreme temperatures and toxicity by chemicals or heavy metals are risks to agriculture (SUBRAMANIAN & CHAREST, 2008; AUDET & CHAREST, 2009) with adverse effects on plant growth and productivity.

The presence of AM fungi in saline soils is common (JUNIPER & ABBOTT, 1993), being able to improve plant growth and production in this condition (DAEI et al., 2009). Strawberry plants are susceptible to salinity (MARTÍNEZ BARROSO & ALVAREZ, 1997), and the use of bio-inoculants in horticultural crops recommended mitigating the negative effects of this stress (SINCLAIR et al., 2014).

The role of these microorganisms to alleviate the damage caused by salinity has been documented in strawberry (FAN et al., 2011; 2012; SINCLAIR et al., 2013; 2014). Mycorrhizal plants are able to increase their tolerance to salinity stress due to their high soil exploration conferred by their hyphae structure and growth

(MIRANSARI et al., 2008). Sinclair et al. (2014) suggest that AM fungi absorb selectively elements such as potassium and calcium, which act as osmotic equivalents, avoiding the absorption of sodium, reducing stress damage.

With changes in the world climate, water limitation is becoming an increasing concern for crop productivity. Strawberry is known to be vulnerable to drought stress (WANG et al., 2008) and its yield and quality are adversely affected (XU & CHEN, 2008).

As previously mentioned, AM fungi improve plant growth and development in different ways. In addition, protect plants against adverse environmental conditions, such as drought (SMITH et al., 2010). Most plant benefit from AM fungi symbiosis under water stress conditions is through the improvement of water status and uptake (AUGÉ, 2001; SMITH et al., 2010).

The presence of AM fungi in strawberry crop was also studied in drought conditions (HERNÁNDEZ-SEBASTIÀ et al., 1999; 2000; BORKOWSKA, 2002; AUGÉ, 2004; BOROWICZ, 2010). Mycorrhization strongly affects growth and tolerance to water deficiency of the plants (BORKOWSKA, 2002). Mycorrhizal fungi influence development of a superior root system, enhance water-conducting capacity, increase uptake of nutrients, and result in higher photosynthetic rates due to better carbon dioxide assimilation (AUGÉ, 2001). Adequate growth in mycorrhizal plants in these conditions can be related to the better use of water in the soil, because the symbiosis provides access to water in soils in conditions below the permanent wilting point (FRANSON et al., 1991) and these changes can be interpreted as drought resistance (PORCEL et al., 2003).

While some earlier reports have successfully demonstrated the usefulness of microorganisms such as a mixture of *Glomus* sp. (BORKOWSKA, 2002), *Penicillium pinophilum* (FAN et al., 2008) and *Glomus mosseae* (YIN et al., 2010) for drought stress tolerance in strawberry. The beneficial effects of inoculation in strawberry under water stress condition are controversial, with positive effect on growth and productivity (BORKOWSKA, 2002; CASTELLANOS-MORALES et al., 2010; FAN et al. 2011; STEWART et al., 2005; YIN et al., 2010; BOYER et al., 2015) and limited or negative responses (GARLAND et al., 2011; VESTBERG et al., 2004; BOROWICZ, 2010).

Along with the increased resistance, the plants have physiological responses such as high stomatal conductance, and consequently the carbon gain (DUAN et al., 1996), water content and maintenance of leaves turgor (AUGÉ et al., 1986). In addition, mycorrhized plants show higher biomass accumulation, larger leaf area, larger root system, these may suggest positive role of AM fungi in protecting photochemical systems against water deficiency (BORKOWSKA, 2002).

2.4 Final consideration

Currently, agriculture requires strategies to reduce inputs, which at the same time ensure crop yield and quality, with a relatively low cost without environmental damage. In this context, an alternative is the use of bio-inoculants such as AM fungi.

Much of what is known regarding the mycorrhizal symbiosis on strawberry crop is resulting from studies in which plants

were grown in sterile soil or substrate. Relatively, little is known about the effect of symbiosis in field conditions where other microorganisms can influence both plant and AM fungi. At the same time, the use of native species or a mix of species as inoculants instead of a single species.

Inoculation with AM in strawberry crop brings varied response because the symbiosis is a result of the interaction between plants, fungi and environmental conditions. In this sense, variation in crop systems, cultivars, crop management and growing conditions can influence these responses. An understanding of the diversity of the soil microorganisms is necessary before soil microbial technologies can be applied.

Although there are such differences responses, literature brings biological, physiological and ecological importance of AM fungi on survival, growth, development and protection of many species of plants. In addition, AM fungi technology playing an important role for the development of sustainable agriculture, mainly by reducing the use of fertilizers and pesticides, which is desired in any farming system, including the strawberry crop.

CHAPTER I

OCCURRENCE AND INFECTIVITY OF ARBUSCULAR MYCORRHIZAL FUNGI IN SOIL UNDER STRAWBERRY CROP

ANA CLÁUDIA PEDERSEN

ABSTRACT - Arbuscular mycorrhizal (AM) fungi form mutualistic relationships with most of terrestrial plant species and are distributed in various habitat types. Considering the presence and diversity of AM fungi in a specific area is the first step for exploiting the benefits of these fungi. The objective of this study was to survey the occurrence of AM fungal species present in soil under strawberry (cv. Camarosa) crop systems. We hypothesized that despite some variability in AM fungal communities across locations some AM fugal species would be commonly found. Soil and roots samples were obtained from five fields under strawberry crops. AM root colonization was determined and soil was chemically characterized. It was also carried out the mycorrhizal inoculum potential (MIP) and soil was used to establish trap cultures to induce sporulation and further identification. All soils had similar number of AM fungi species; however, each site presented some different species. Including all five soil, thirteen Glomeromycota species were identified. AM root colonization and MIP values also varied among the sites. Arbuscular mycorrhizal fungi diversity, root colonization and inoculum potential in the rhizosphere of strawberry crops shows differences among the sites surveyed

regardless the same type of soil under the same cultivar, indicating that other factors may be influencing the establishment of these species. The diversity may be related to the variability among the sites. The two most frequent species in soils under strawberry crop are *Claroideoglomus etunicatum* and *Funneliformis mosseae*.

Key words: *Fragaria* x *ananassa* Duch., inoculum, mycorrhizal colonization, potential, soil characteristics, species.

OCORRÊNCIA E INFECTIVIDADE DE FUNGOS MICORRÍZICOS ARBUSCULARES EM SOLO SOB CULTIVO DO MORANGUEIRO

RESUMO - Fungos micorrízicos arbusculares (MA) formam relações mutualistas com a maioria das espécies de plantas terrestres e estão distribuídos em vários tipos de habitats. Considerar a presença e diversidade de fungos em uma área específica é o primeiro passo para a exploração dos benefícios destes fungos. O objetivo deste trabalho foi determinar a ocorrência de espécies de fungos MA presentes em solo sob cultivo de morangueiro (cv. Camarosa). Hipotetizamos que, apesar de alguma variabilidade na comunidade de fungos MA entre os locais algumas espécies seriam comumente encontradas. As amostras de solo e raízes foram obtidos a partir de cinco áreas de cultivo do morangueiro. A colonização micorrízica das raízes foi determinada e o solo foi caracterizado quimicamente. Também foi determinado o potencial inóculo do solo (MIP) e culturas armadilhas foram estabelecidas para induzir a esporulação e posterior identificação a

nível de espécie. Todos os solos tiveram número similar de espécies identificadas, no entanto, cada local apresentou algumas espécies em particular. No total, treze espécies de Glomeromycota foram identifacadas. A colonização micorrízica e MIP também variaram entre os locais. Diversidade de fungos, colonização micorrízica e potencial inóculo mostram diferenças entre os locais pesquisados independentemente do mesmo tipo de solo sob a mesma cultivar, indicando que outros fatores podem estar influenciando o estabelecimento dessas espécies. A diversidade pode estar relacionada com a variabilidade entre os locais. As duas espécies mais frequentes em solos sob cultivo de morangueiro são *Claroideoglomus etunicatum* e *Funneliformis mosseae*.

Palavras-chave: *Fragaria* x *ananassa* Duch., inóculo, colonização micorrízica, potencial, características do solo, espécies.

1 INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are important soil dwelling organisms because they are widely distributed and form mutualistic associations with the roots of most terrestrial plant species (SCHÜβLER et al., 2001; SMITH & READ, 2008). The host plants provide carbon compounds to AM fungi primarily in exchange for inorganic minerals (TOMÉ et al., 2015).

The diversity of AM fungal spores is context dependent and results from complex fungus x plant host x fungal community x soil micro(biota) x abiotic environmental conditions. Agricultural

practices have a significant impact on these interactions (SIEVERDING, 1989; OEHL et al., 2003; MOREIRA & SIQUEIRA, 2006) and different species of AM fungi differ in their tolerance to these practices (KUMAR & GHOSE, 2008). Moreover, plant species also vary in their symbiotic responsiveness to AM fungal communities with respect to plant growth and fitness (DANESH et al., 2006). As such, in order to maximize the potential benefits resulting from the AM symbiosis, it is important to first determine patterns of AM fungal diversity in specific crops in a given soil type and under a range of standard set of management practices for that crop.

Strawberry (*Fragaria* x *ananassa* Duch.) is one of the most intensively produced fruit crops globally. Global strawberry production is twice the amount of all other berry crops combined (STEWART, 2011). It is a crop of great economic and social importance in several countries. The five top producers are China, United States, Mexico, Turkey and Spain. From 2010 to 2013 world production increased 17.4%, and the highest yield was obtained in the United States, 58 t ha-1, followed by Mexico, 45 t ha^{-1} (FAOSTAT). In Latin America, Chile is the largest producer, followed by Brazil (OLIVEIRA & SCIVITTARO, 2009). Strawberry crops are an important socio-economic activity in many regions of Brazil. National production is approximately 100,000 tonnes per year in an estimated area at 3,500 ha (CARVALHO, 2006; ANTUNES & REISSER JÚNIOR, 2007), mostly from soil cultivation (CARVALHO, 2006). These numbers are expected to increase with the incorporation of new technologies and cultivars (OTTO et al., 2009).

Strawberry is highly responsive to AM fungi (VESTBERG et al., 2004; MALUSA et al., 2007). However, inoculated strawberry plants show contrasting responses in relation to yield, with positive (SHARMA & ADHOLEYA, 2004; DOUDS et al., 2008) and no responses observed (KHANIZADEH et al., 1995) depending on cultivar and/or AM fungal isolate (STEWART et al., 2005), environmental conditions (BULL et al., 2005) and inoculum quality (NIEMI & VESTBERG. 1992).

Species diversity is an important factor affecting plant biodiversity and ecosystem productivity (van der HEIJDEN et al., 1998). Therefore, knowledge of the indigenous diversity of AM fungi in specific strawberry cropping systems is an important first step to determine the potential for this crop to benefit from the symbiosis. Based on this knowledge we may be able to fine tune agricultural practices that take greater advantage of indigenous AM fungal communities and potentially select isolates capable of providing most benefit to strawberry while tolerating the agronomic practices required by this crop (DOUDS & MILLNER, 1999; BRITO et al., 2008). Furthermore, this can lead to the establishment of alternative management methods that reduce inputs (SILVEIRA, 1998).

AM fungal diversity differs among ecosystems and is typically greater in natural compared to agricultural ecosystems (WANG et al. 2003). In addition, AM fungi differ between propagule forms such as roots, spores and soil mycelium (CERVERO et al., 2015). Furthermore, AM fungi are particularly important in tropical regions where soils usually present low fertility than at higher latitudes (JANOS, 1996) and richness of AM fungal communities

decreases with latitude (DAVISON et al., 2015). Souza et al. (2010) recorded 119 species, from 163 surveys in different regions and ecosystems in Brazil, representing 55% of the known global diversity. From the 26 states and Federal Districts only 8 states have no records of AM diversity, mostly northern states. In addition, the authors reported that states with the highest number of surveys are not necessarily those with the most richness of species. This indicates that Brazilian ecosystems are important sources of AM fungi diversity (STÜRMER & SIQUEIRA, 2006; 2008), which may present much potential for applications.

Identification of AM fungal spores collected directly from soil or through trap cultures is a relatively inexpensive and simple method of evaluating the AM fungal diversity because spores have morphological characteristics required for their distinction (MORTON et al., 1995, STÜRMER & SIQUEIRA, 2008). Furthermore, phylogenies using rDNA have generally supported the earlier approaches using spore morphology (REDECKER et al., 2000; SCHÜβLER et al., 2001). Since field collected spores are in some cases found in low numbers, parasitized and lacking informative taxonomic characteristics (CLAPP et al., 1995), establishment of trap cultures is a strategy that can yield a large number of healthy spores and, therefore, facilitate identification (LEAL et al., 2009). Nevertheless, trap cultures do not necessarily allow the identification of all species, because AM fungal sporulation is affected by the host species used in the trap culture (BEVER et al., 1996). However, in some cases trapping promotes the sporulation of cryptic AM fungal species that were not sporulating in the field (STÜRMER, 2004;

OEHL et al., 2004). Also, determine the mycorrhizal inoculum potential, i.e. estimation of all viable propagules responsible for the initial infection in the root systems of host plants, is a parameter used to assess the quality of the soil (LIU & LUO, 1994), since AM fungi are more abundant where plants are more limited by soil nutrients (TRESEDER, 2004). Evaluation of AM fungal spores as well as colonization is therefore important to know the level of its association because of the differences in growth and sporulation among AMF species (LAND & SCHÖNBECK, 1991).

We hypothesized that AM fungi communities associated with strawberry plants are similar across sites under cultivation of the same strawberry cultivar. The objective of this study was to survey the occurrence of AM fungal species present in soil under different continuous (i.e., more than three years) strawberry crop systems.

2 MATERIAL AND METHODS

2.1 Study site and sample collection

The sites were located in northwest Rio Grande do Sul (RS) state in Brazil, at Ibirubá, Tapera and Passo Fundo cities. Samples were collected at the end of the strawberry season, in January 2014. The study focused on the most cultivated strawberry cultivar (Camarosa) in RS cultivated on Oxisol under a variety of management practices (Table 1).

Soil samples were collected along the plant rows with a core (10 cm diameter) at a depth of 5-20 cm from five different points in each study site. At each location we collected five cores within an

area of 1 m^2 and pooled the cores in polyethylene bags, totalling five samples per site. Approximately 1 kg of rhizosphere soil was collected in each location and a portion of this soil was used to establish trap cultures for subsequent spore extractions for identification. AM fungal community composition was assessed among locations by spore extraction. We conducted five extractions per site, from 50 g of soil each, and all spores were pooled together for further identification. The remaining soil was used to determine mycorrhizal inoculum potential (MIP) and to assess soil fertility following the methods of Tedesco et al. (1995).

Table 1. Study sites surveyed in Rio Grande do Sul, Brazil. UPF, Passo Fundo, 2014

Characteristics	Sites				
	P1	I1	I2	T1	T2
Coordinates	28°15'41"S 52°24'45"W	28°37'04"S 53°08'25"W	28°36'08"S 53°01'05"W	28°37'23"S 52°52'00"W	28°38'18"S 52°52'05"W
Elevation (m)	687	416	416	409	409
Cultivar	Camarosa	Camarosa	Camarosa	Camarosa	Camarosa
Mulching	Plastic	Plastic	Organic matter	Organic matter	Plastic
Field	High tunnel	Sombrite	Open filed	Low tunnel	Low tunnel
Fertilizer	Mineral	Mineral	Organic	Organic	Mineral

2.2 AM root colonization

At the same locations of soil collection five strawberry plants were collected to estimate AM fungal root colonization. Root samples were washed thoroughly with running tap water to remove the adhered soil particles and cut into small pieces of about 1 cm according to Phillips & Hayman (1970). AM root colonization was quantified placing 1 cm segments onto a microscope slide and observed under microscope (TROUVELOT et al., 1986). Accordingly, 100 intersections were quantified for each sample and

the presence of any vesicles, arbuscules, or internal hyphae was assessed and percent root colonization was calculated from the number of colonized intersections out of total root intersections.

2.3 Mycorrhizal inoculum potential

To determine AM fungal inoculum potential of soils from the different locations, a greenhouse experiment was conducted following methodology by Moorman & Reeves (1979). Soil samples from each site were diluted with a base of sterilized (121ºC for 60 min) sandy loam soil (1:1, v:v) in two dilution series of the original soil (1:1 and 1:4, v:v) and inoculum potential was estimated at 30 and 60 days after sowing. There were five replicates per dilution and evaluation time per location. *Sorghum sudanense* was sowed into plastic containers-tubes (290 mL). Upon seedling establishment, plants were thinned to one per tube. Plants were watered daily without fertilizer application.

After 30 and 60 days of growth, roots were harvest to determine AM inoculum potential through AM root colonization (as described earlier).

2.4 Trap culture

Trap cultures were established to trigger sporulation of indigenous AM fungi and to obtain clean material. Traps were established and let to grow for five months under greenhouse conditions. They were established by placing 200 g of field collected soil (containing root pieces) layered between two layers of sterilized sand (two cycles of 121ºC for 60 min, 24h interval), in 1.0 kg plastic

pots. Approximately 60 seeds of *S. sudanense*, were sown at 2 cm depth in each pot and covered with sterilized sand. Pots were irrigated daily and no fertilization was added during the growth period. After five months of growth, 50 g soil was sampled from each pot for AM fungal spore extraction and identification.

2.5 Spore extraction and identification

Arbuscular mycorrhizal fungal spores were extracted by the wet sieving method described by Gerdemann & Nicolson (1963) followed by sucrose gradient centrifugation (20% and 60%). Under the microscope, spores were separated by morphotypes and were mounted on slides in PVLG and PVLG+Melzer´s reagent. Spores were crushed by applying pressure to the cover slip and then stored at 65ºC for 24 h to clear their contents from oil droplets before being identified based on subcellular structures following descriptions of living reference cultures (http://invam.caf.wvu.edu) and their classification according to Redecker et al. (2013).

2.6 Statistical analysis

Mycorrhizal root colonization and mycorrhizal inoculum potential were set up in a completely random design, with five replications, considering sites as treatments. Percentage data were transformed to arcsin (x/100) followed by variance analysis (ANOVA) and treatment means were compared using Tukey’s test, 0.05 significance level. Pearson’s correlation coefficients were calculated between chemical soil properties and the AM root colonization and inoculum potential.

3 RESULTS

The soil samples even belong to the same taxonomy group (Oxisol) had different chemical characteristics, especially pH, phosphorous (P) content and organic matter (Table 2), probably due to the cropping management used, especially fertilizer application (Table 1).

Table 2. Soil chemical characteristics. UPF, Passo Fundo, 2014

Soil properties	Sites				
	P1	I1	I2	T1	T2
Clay (%)	15.5	34.7	25.8	28.0	23.7
pH H_2O	6.2	7.0	6.2	6.6	5.8
Index SMP	7.0	6.9	6.3	6.5	6.0.
O.M. (%)	4.1	3.1	4.7	3.2	2.5
P (mg dm^{-3})	134.4	48.3	324.0	172.7	211.0
K (mg dm^{-3})	682	426	630	637	435
Al (cmolc dm^{-3})	0	0	0	0	0
Ca (cmolc dm^{-3})	9.5	8.3	7.8	7.9	5.1
Mg (cmolc dm^{-3})	4.9	3.3	3.0	1.9	1.6
H+Al (cmolc dm^{-3})	1.4	1.6	3.1	2.5	4.4
CEC (cmolc dm^{-3})	17.5	14.2	15.5	13.8	12.2
Saturation (%)					
Base	92	89	80	82	64
Al	0	0	0	0	0
K	9.9	7.7	10.4	11.8	9.1

P: phosphorous; K: potassium; O.M.: organic matter; Ca: calcium; Mg: magnesium; AL: aluminium; H+Al: potential acidity; CEC: cationic exchange capacity.

Table 3. Arbuscular mycorrhizal fungi species identified from five site of strawberry crop. UPF, Passo Fundo, 2014

AM fungi species	Sites				
	P1	I1	I2	T1	T2
Paraglomerales					
Paraglomeraceae					
Paraglomus brasilianum	-[1]	-	+	-	+
Glomerales					
Claroideoglomeraceae					
Claroideoglomus claroideum	-	-	-	+	+
Claroideoglomus etunicatum	+	+	-	+	+
Glomeraceae					
Rhizophagus intraradices	-	-	+	-	-
Glomus sp1	-	-	+	-	-
Glomus sp2	-	+	-	+	-
Glomus sp3	-	-	-	-	+
Funneliformis mosseae	+	+	+	+	-
Septoglomus viscosum	+	+	-	-	-
Diversiporales					
Acaulosporaceae					
Acaulospora mellea	+	-	+	-	+
Acaulospora morrowiae	+	-	-	-	+
Acaulospora scrobiculata	-	+	-	-	-
Gigasporaceae					
Cetraspora pellucida	+	-	-	-	-
Mycorrhizal colonization (%)	53.8 a[2]	51.2 ab	11.6 b	58.0 a	56.4 a

[1](-) absence or (+) presence of arbuscular mycorrhizal fungi species.
[2]Means followed by the same lower case letters in the column do not differ significantly by the Tukey's test ($p \leq 0.05$).

A total of five families of Glomeromycota were represented in trap cultures and 13 species were identified (Table 3), but three species could not clearly be identified at the species level

bellowed to the genus *Glomus*. It was observed that *Claroideoglomus etunicatum* and *Funneliformis mosseae* were present in most of the sites. For instance, it is noteworthy species belonging to the family Glomeraceae were the predominant sporulators in trap cultures and also presented the highest number of species.

AM fungi colonized all plants samples and formed arbuscular mycorrhizal structures in the root tissues and varied among sites (Table 3).

The values of AM root colonization to determined MIP varied from 12 to 42% after 30 days and 25 to 68% after 60 days regardless dilution. MIP was higher for all sites after 60 days, without differences between the dilutions. The site I2 had the lowest value of MIP (Table 4).

Table 4. Mycorrhizal inoculum potential of five soils from strawberry farms in Rio Grande do Sul, Brazil. UPF, Passo Fundo, 2014

Sites	Mycorrhizal inoculum potential			
	Dilution (soil:diluent)			
	1:1 (v:v)		1:4 (v:v)	
	Evaluation time (days)			
	30	60	30	60
P1	42.00 ± 22.67	61.60 ± 18.40	12.00 ± 8.94	52.00 ± 31.62
I1	36.40 ± 11.87	47.20 ± 14.11	33.20 ± 29.00	68.00 ± 16.79
I2	12.00 ± 7.21	25.60 ± 6.84	12.40 ± 6.23	30.00 ± 17.03
T1	41.20 ± 21.70	64.40 ± 18.08	20.80 ± 22.12	48.00 ± 11.74
T2	25.20 ± 12.38	34.40 ± 16.46	20.00 ± 9.38	48.00 ± 17.38

4 DISCUSSION

Our main goal was to determine whether or not AM fungal community composition varied with location when host variety and soil type were kept constant but other factors, including management practices, varied. We detected clear differences in AM fungal community composition among the sites. Oehl et al (2010) showed that soil types and land use intensity must differ in the AM fungal community.

Species from Glomeraceae and Acaulosporaceae family are dominated among the 13 species described. In RS, the dominance of the genus *Glomus* followed by *Acaulospora* was also observed in crops such as grapes (ÁVILA, 2004), citrus (SOUZA et al., 2002; FOCCHI, 2003), peach (NUNES, 2007).

It is no surprise that most of AM fungi species owned to the family Glomeraceae because this is the prevailing family in agricultural soil (SOUZA et al., 2010). The *Glomus* genus presents greater ability to adapt to soils subjected to fertilization, liming and cultivation practices (CARRENHO et al., 1998) and propagates in systems with different environmental degradation levels (BAREA et al., 2011).

All AM fungi species found in our study had been previously record in Brazil, but not in RS, *S. viscosum*, *P. brasilianum* and *R. intraradices* (SOUZA et al., 2010). These authors report, from systematic review, that RS has one of the most richness describe in Brazil with 50 species, from a total of 119 species, identified in different ecosystems, including agricultural systems. Species from Glomeraceae and Acaulosporaceae were dominant in all Brazilian

ecosystems, as well as in this study. However, RS has been poorly investigated and little is known about AM diversity.

Crop management practices can influence soil chemical characteristics, and the question is whether the occurrence of specific AMF species was determined by those properties. There is a difficulty of establishing a standard for the occurrence of AM fungi, as this may be associated with various factors related to the environment, as well as different fungal survival strategies. Communities of AM fungi is highly influenced by specific factors including climatic conditions, soil physic-chemical status, variety of host plant and agricultural practices (SMITH & SMITH, 1996; OEHL et al., 2003, 2005; GOSLING et al., 2006; HELGASON & FITTER, 2009). Furthermore, different species of AM fungi differ in their tolerance to adverse physical and chemical conditions in soil (KUMAR & GHOSE, 2008). Some studies have showed that pH (EZAWA et al., 2001; OEHL et al., 2010), nitrogen (JOHNSON et al., 2003; EGERTON-WARBURTON et al., 2007), organic matter (BODDINGTON & DODD, 2000) and phosphorous availability (OEHL et al., 2004) influence the composition of AM fungi communities.

The occurrence of exclusive species suggests affinity of these AM fungi species with specific conditions of the sites. We observed that two species (*C. etunicatum* and *F. mosseae*) were present in four of the five sites, except at I2 and T2, respectively. Some species can be adapted in some soil. These agriculturally adapted AM fungi species have slower rates of infection, are faster to sporulate and to produce fewer arbuscules (OEHL et al., 2003). This adaptability can reduce the rates of infection as observed in site I2

(11.6%) in a soil with high frequency of the Glomeraceae family. Also, monocultures appear to change AM species composition and reduce their population diversity (VESTBERG et al., 2005). This way, it is possible suggest crop rotation as a management practice, using mycorrhizal host plants species, to increase the number of AM fungi species in the soil and prevent the adaptation of some species.

Only one member of the Gigasporaceae family was identified in this survey, and also only in one site. This site is characterized by production in high tunnels and tillage is not done routinely. As reported by the farmers, before transplanting strawberry plantlets, the soil is tillage, and this practice can affect the AM root colonization due to rupture of the hyphal network (JASPER et al., 1989; GOSS & de VARENNES, 2002). Member of Glomeraceae have a highly infective extra-radical mycelium that could allow colonising immediately plant root (HART & READER, 2002). Contrary, members of Gigasporaceae are only capable of propagation via spore dispersal or infection from an intact mycelium (INVAM).

Our results showed that the AM root colonization rates were in the same range as previous studies with strawberry, such as 25 to 75% (CHAVÉZ & FERRERA-CERRATO, 1990), except the site I2 (Table 3). AM root colonization is determined by the plant species that are present at the site and edaphic factors (AHULU et al., 2006; CARRENHO et al., 2007). Since the plant species was not an experimental variable in our study, the reason for similarities and differences of AM root colonization and also AM fungi species should be based on other variables among the sites such as crop history, crop management practices and soil chemical characteristics.

There is much evidence supporting that soil properties have a large influence on AM fungi (JOHNSON et al., 2005; CARRENHO et al., 2007; GRYNDLER et al., 2009). The decrease in AM root colonization with an increase in soil P content observed in the study can be attributed to the fact that high fertility reduced AM root colonization (KAHILOUTO et al., 2001). The roots with lowest mycorrhizal colonization were grown in a soil with high organic matter (Table 2). Organic matter does not always explain the mycorrhizal development in the roots (SYLVIA & WILLIAMS, 1992). However, combined with P content it is possible to observe the effect on AM root colonization. High OM e high P content decreased AM colonization although the number of AM species did not change. This shows that the fertilizer management can influence the soil characteristics and hence negatively impact the growth and development of the plant.

Mycorrhizal inoculum potential (MIP) was significantly higher in P1 and T1 compared to I2 (Table 4). The highest values found in soils from these areas may be related to the characteristics of each site. Phosphorous content has an inverse relation to mycorrhizal dependence (SIQUEIRA & COLOZZI-FILHO, 1986) for restricting mycorrhizal infection and reducing the percentage of colonized roots (MELLONI et al., 2000; NOGUEIRA & CARDOSO, 2000). Consequently, reduce the fungi propagules and inoculum potential.

This variable provides information about the activity of the AM fungal community in the soil (STÜRMER et al., 2013). This way, this results indicated that in most areas assessed the AM propagules are active and at appropriate levels to establish the

symbiosis in a short period of time. It is also observed that MIP is not related to the number of species present in each site, since all areas showed a similar number of AM species (Table 3).

An explanation for contrasting AM root colonization values, inoculum potential and species among the sites may be the differences in the selective pressures imposed by anthropogenic impacts, in particular differences in the use of fertilizers which have a selective effect on AM fungi (GOSLING et al., 2006).

Horticultural systems are often fertilized with high amounts or in contrast with reduced amounts of fertilizer, even pesticides, depending on the economic situation of the small farmers and the crop system used. Describing the AM fungi community in different sites is an important step in determining the effects of agriculture managements upon AM fungi and the eventual development of practices for these soil microorganisms (DOUDS Jr. & MILLNER, 1999).

This study on the diversity of AM fungi is the first survey of these soil microorganisms associated with strawberry plants in Brazil. Our results contribute to the knowledge AM fungi species occurrence in this crop, even our results are based exclusively on trap cultures spores and only five strawberry farms, which may not represent the entire AM fungi community. Further studies should assess AM fungi diversity in other strawberry crop regions and systems in RS and Brazil. However, further AM diversity studies should consider molecular techniques considering that some studies cannot reach the specie level, especially for species belonging to the genus *Glomus*.

Despite the improper management practices, our study reveals that soil from these regions have moderate inoculum potential as well as AM fungi species diversity. This provides great possibilities for further studies and utilization of AM fungi in horticulture systems. Thus, agricultural management practices should aim to increase the soil microbiological resources as AM fungi communities and its functionality aiming high yield with lower input.

5 CONCLUSIONS

Occurrence of arbuscular mycorrhizal fungi, root colonization and inoculum potential in the rhizosphere of strawberry crops shows differences among the sites surveyed. Among the thirteen species described the two most frequent species in soils under strawberry crop are *Claroideoglomus etunicatum* and *Funneliformis mosseae*.

CHAPTER II

ARBUSCULAR MYCORRHIZAL FUNGI INOCULATION ON STRAWBERRY GROWTH AND YIELD

ANA CLÁUDIA PEDERSEN

ABSTRACT - Crop management practices that aim greater fruit yield and quality with lower environment impact is required. A strategy able to contemplate these three aspects is the use of beneficial soil microorganisms as arbuscular mycorrhizal (AM) fungi. A greenhouse experiment was carried out to evaluate the effect of AM fungi inoculation with both indigenous species and single-specie, on strawberry growth, development and yield. Strawberry plantlets (cv. Camarosa) were transplanted in a soilless system. The experimental design was a split-plot design with four replications. The treatments consisted in inoculum (native community, isolate 1 (*C. etunicatum* SCT101A), isolate 2 (*C. etunicatum* MGR288A-1) and control) and along crop season (blooming, fructification and end of the season). There is interspecific variability among AM inoculum on the capacity to colonize the roots, without interference of the phenological phases. The presence of the AM inoculum during transplanting do not resulted in precocity of strawberry. Inoculation with AM fungi in strawberry plants (cv. Camarosa) affects the leaf appearance rate and phyllochron with different responses depending on the development stage. However, did not modify biomass production, weight and diameter of strawberry fruit and yield growth in a soilless system.

Key words: biomass, endomycorrhiza, *Fragaria* x *ananassa* Duch., fruit, inoculation, precocity.

INOCULAÇÃO COM FUNGOS MICORRÍZICOS ARBUSCULARES SOBRE O CRESCIMENTO E PRODUÇÃO DE MORANGUEIRO

RESUMO - A utilização de práticas de manejo que visam maior produtividade e qualidade dos frutos, juntamente com menor impacto ao meio ambiente, é desejável. Uma ferramenta capaz de comtemplar estes três aspectos no cultivo do morangueiro é o emprego de microrganismos benéficos presentes no solo, como os fungos micorrízicos arbusculares (MA). Um experimento em uma estufa plástica foi conduzido para verificar se há interferência do tipo de inóculo micorrízico sobre o crescimento, desenvolvimento e rendimento do morangueiro, no sistema de cultivo sem solo. Mudas de morangueiro (cv. Camarosa) foram transplantadas para um sistema de cultivo sem solo. O delineamento experimental foi em esquema de parcelas subdivididas com quatro repetições. Os tratamentos consistiram de inóculo (comunidade nativa, isolado 1 (*C. etunicatum* SCT101A), isolado 2 (*C. etunicatum* MGR288A-1) e controle) edurante o ciclo da cultura (floração, frutificação e no final do ciclo). Concluiu-se que há variabilidade interespecífica no fungo micorrizico arbuscular quanto à capacidade de colonização das raízes, sem interferência das fases fenológicas. A presença da micorriza durante o transplante não resultou em precocidade do morango. A inoculação

com fungos MA em plantas de morango (cv. Camarosa) afeta a taxa de aparecimento de folhas e filocrono com respostas diferentes dependendo do estágio de desenvolvimento. Entretanto, não há interferência da inoculação sobre a produção de biomassa, peso e diâmetro dos frutos e na produtividade do morangueiro sob sistema de cultivo sem solo.

Palavras-chave: biomassa, endomicorriza, *Fragaria* x *ananassa* Duch., frutos, inoculação, precocidade.

1 INTRODUCTION

Strawberry (*Fragaria* x *ananassa* Duch.) is one of the most intensively produced fruit crop. This berry is cultivated around the world and is appreciated, not only because of its characteristic aroma, bright red color, juicy texture and sweetness, but also for its nutritional value (MANDAIL, 2009). World production increased 17.4% from 2010 to 2013. Improved production technology have contributed to greater yields and improved product quality (NACIMIENTO, 2009; VICENTE et al., 2009; ANTUNES et al., 2010). Currently, it is estimated that South America produces 318,686 tons of strawberries on 11,884 hectares; Brazil, Argentina and Chile are responsible for most of the production (ANTUNES & PERES, 2013). In Brazil, production is around 100 thousands tones in an estimated area at 3,500 ha (CARVALHO, 2006; ANTUNES & REISSER JÚNIOR, 2007). The principal producing states are Rio Grande do Sul (25.6%), São Paulo (15.4%), and Minas Gerais

(41.4%) (OLIVEIRA et al., 2009), but strawberry production is increasing in other regions and states (ANTUNES & PERES, 2013). This yield tends to increase as the incorporation of new technologies and cultivars (OTTO et al., 2009).

The use indiscriminate of products to control plants, improperly nutritional management during the cultivation period, and other practices, have negative impacts on the environment. Moreover, population requires an increase in yield and/or quality.

Crop management practices that aim greater fruit yield and quality with lower environment impact is required. A strategy able to contemplate these three aspects is the use of beneficial soil microorganisms as arbuscular mycorrhizal (AM) fungi, biotrophics, able to colonized the roots and establish a symbiosis with most of vascular plants (SMITH & READ, 2008). These symbioses promote plant growth by enhancing nutrient uptake and protection against biotic and abiotic stresses (KHANIZADEH et al., 1995; POZO & AZCÓN-AGUILAR, 2007; LOWE et al., 2012).

Strawberry is highly responsive to colonization with AM fungi (BORKOWSKA, 2002; DOUDS et al., 2008; CASTELLANOS-MORALES et al., 2010). It has been shown that some arbuscular mycorrhizal fungi can positively affect the growth and development of strawberry (KIERNAN et al., 1984; CHANG, 1986; VOSATKA et al., 1992; VESTBERG et al., 2004; MALUSA et al., 2007).

Therefore, it is expected that inoculation with MA fungus can have a positive effect on precocity, determined by estimating the leaf appearance rate (LAR) and phyllochron. LAR is represented by the number of leaves on the main stem (STRECK et al., 2003a) and

phyllochron is the time between the appearances of two successive leaves on the main stem (KLEPPER et al., 1982). LAR affects the evolution of leaf area index (LAI), which is responsible for intercepting solar radiation necessary to crop photosynthesis, which ultimately defines crop growth and yield (STRECK et al., 2003b). Information relating to phyllochron is poorly known, despite their importance for plant growth and development models, whose answers are able to support crop management practices, sowing dates and most suitable regions for the cultivation (DALMAGO, et al., 2013). Studies have documented a linear response of leaf number and temperature in strawberry in different growth systems and cultivars (ROSA et al., 2011; MENDONÇA et al., 2012; COSTA et al., 2014; TAZZO et al., 2015). However, there are no reports of mycorrhizal fungi inoculation in the leaf appearance and phyllochron in strawberry.

Strawberry plant growth and fruit production increased following the AM fungi inoculation (DOUDS et al., 2008). Among the various factors that contribute the growth and yield of strawberry, nutrition is the important for crop production (UMAR et al., 2008). Positive growth response to mycorrhizal fungi and increase in nutrient uptake has also been reported in strawberry crops (HOLEVAS, 1966; DUNNE & FITTER, 1989; HRSELOVA et al., 1990; TAYLOR & HARRIER, 2001). Nevertheless, the same can be observed in different combination between strawberry cultivars and AM fungi species (ROBERTSON et al., 1988; CHAVEZ & FERRERA-CERRALO, 1990; WILLIAMS et al., 1992; NIEMI & VESTBERG, 1992; VARMA & SCHUEPP, 1994).

Inoculated strawberry plants present divergent responses in relation to yield, with positive (BORKOWSKA, 2002; SHARMA & ADHOLEYA; 2004, DOUDS et al., 2008) and negative reports (NIEMI & VESTBERG, 1992; KHANIZADEH et al., 1995; BULL et al., 2005). These different responses may be related to the use of AM fungi species in commercial products, which are not present in cultivated soil, leading to competition between native and introduced species. Locally AM fungi isolates can be produced on farm (DOUDS et al., 2008) and may be more effective than introduced species (BULL et al., 2005) for being more efficient as they are locally adapted to the soil conditions (SREENIVASA, 1992; OLIVEIRA et al., 2005b). This way, it may be better to promote practices that enhance native AM diversity than to introduce inoculants based on one AM species (GARLAND et al., 2011).

AM fungal species are functionally different and their impact on a host plant may be complementary (HART & KLIRONOMOS, 2003). A diversity of AM fungal species (indigenous or exogenous) may allow AM fungal populations to better adapt to fluctuating environmental conditions and achieve a higher consistency in the plant responses (KOOMEN et al., 1987). Inoculation with indigenous fungal populations usually provides the most satisfying results, due to the opportunity to find compatible symbionts and to enhance the mutualistic effects with two or more symbionts, rather than just with one (REGVAR et al., 2003). Taylor & Harrier (2001) and Khanizadeh et al. (1995) found that strawberry vegetative growth response to inoculation depended on cultivar-AM species combinations. This study shows that host plants have high

selectivity for AM fungi and the fungi have high host specificity and local diversity.

In addition, the AM fungi play an important role for the development of sustainable agriculture, mainly by reducing the use of fertilizers and pesticides, which is desired in any farming system, including the strawberry crop. Studies have shown that this may be a potential strategy of biofertilization to increase yield, enhance compounds that represent the quality and nutraceutical product content and also make plants more resistant (SHARMA & ADHOLEYA, 2004; MATSUBARA et al., 2009).

Field experiments have focused on agricultural monocultures and prevalent species of AM fungi (e.g. *Rhizophagus intraradices* reported as *Glomus intraradices*) (LEKBERG & KOIDE, 2005). This study aimed check for interference on the type of mycorrhizal inoculum on strawberry growth, development and yield growth under soilless system during crop season.

2 MATERIAL AND METHODS

2.1 Plant material and mycorrhizal inoculum

From June to December 2014, an experiment was carried out in a greenhouse in Passo Fundo/RS/BR (28º15'41" S, 52º24'45" W, elevation 709 m). It was used bare roots plantlets of strawberry (cv. Camarosa) from Agrícola LLahuen nursery, Chile (33º50'30.19" S, 70º40'05.04" W), provided by Strawberry Breeding Program at the University of California. The plantlets production is performed at open field after soil sterilization. After two months the commercial

plantlets are harvest mechanically and prepared by washing and selected according to the crown diameter. The plantlets are packed leafless and sent to Brazil in containers and delivered after 10-12 weeks.

Field soil from a strawberry crop (cv. Camarosa) at Universidade de Passo Fundo (28º15'41"S, 52º24'45"W, 709 m) was collected in January 2014 to use as an inoculum containing a native community. Others soils from a strawberry crop were tested. As criteria for choosing the soil used as inoculum was sporulation in the trap culture and mycorrhizal inoculum potential.

Trap cultures were established to trigger sporulation of native AM fungi species. Traps were set up and let to grow for 18 weeks under greenhouse conditions at University of Passo Fundo. Traps were established by placing 200 g of soil sample, containing strawberry root pieces, as layers between two layers of sterilized sand (121 ºC, 1h, two cycles with 24h interval), in 1.0 kg plastic pots. Approximately 60 seeds of *Sorghum sudanense*, as a host plant, were sown at 2 cm depth in each pot and covered with sterilized sand. Pots were irrigated daily and no fertilization was added during the growth period. After 18 weeks of growth, 50 g soil was sampled from each pot for AM fungi spore extraction and identification. Arbuscular mycorrhizal fungi spores were extracted by the wet sieving method described by Gerdemann and Nicolson (1963) followed by sucrose gradient centrifugation (20% and 60%). AM fungi species were identified, at University of Blumenau-Brazil, based on subcellular structures of asexual spores following description of living reference cultures (http://invam.caf.wvu.edu) and their classification according

to Redecker et al. (2013). Also was used two AM fungi isolated specie as inoculum (*C. etunicatum* SCT101A and *C. etunicatum* MGR288A-1), originally isolated from Brazil, obtained from Coleção Internacional de Cultura de Glomeromycota (CICG; http://www.furb.br/cicg).

Annual average of accumulated hours of chill is 422 hours, with lower or equal temperature to 7.0ºC ranging from 214 to 554 hours (CUNHA, 2003).

2.2 Treatments and experimental design

The treatments consisted in inoculum and phenological stages. Inoculum: (1) control; (2) indigenous community consisting of *Acaulospora mellea*, *A. morrowiae*, *Cetraspora pellucida*, *Claroideoglomus etunicatum*, *Glomus* sp. and *Septoglomus viscosum*; (3) Isolate 1 - *C. etunicatum* SCT101A; and (4) Isolate 2 - *C. etunicatum* MGR288A-1. Phenological stages: blooming; fructification; and, end of the cycle.

The treatments were distributed in a split-plot design with four replicates. In the main plot was tested the type of inoculum and in the split-plot was tested the phonological stages. It was transplanted four plants per plot, all plants were evaluated. The plant population was 16 plants m^{-2}.

2.3 Experiment setup

Bare root plantlets of cv. Camarosa, were transplanted in white low-density polyethylene bags (150 µm thickness) tubing shaped (0.5 m length and 0.3 m width), placed on wood stand. The

bags were filled with 20 dm^3 of autoclaved sand (121 ºC, 1h, two cycles with 24h interval) and four plantlets were transplanted per bag. Transplant was done on June 17th, 2014.

A drip-irrigation system was placed into the bags near each plant. Irrigation pulses with duration of 5 min were applied (at a volume of 0.35 L per pulse per plant) every day. The nutrient solution recommended by Calvete et al. (2007) was used for fertirrigation, once a week. The pH and electrical conductivity (EC) of the fertilizer solution and the solution drained by the bags was monitored. The fertilizer solution pH and EC range from 5.5 to 6.7 and 1.15 to 2.15 dS m^{-1}, respectively. The drain solution pH did not vary and EC range from 0.1 to 1.7 dS m^{-1}, among the treatments.

Temperature and photosynthetically active radiation (Figure 1) were measured inside the greenhouse using a weather Station (WatchDog), during the experiment.

Autoclaved sand were analysed for chemical characteristics (TEDESCO et al., 1995). The chemical analysis of the sand showed the follow values for cation exchange capacity (7.8 $cmol_c$ dm^{-3}), pH (5.7), phosphorous (9.9 mg dm^{-3}), potassium (53 mg dm^{-3}), calcium (2.7 $cmol_c$ dm^{-3}), magnesium (1.1 $cmol_c$ dm^{-3}), sulfur (5 mg dm^{-3}), boron (0.1 mg dm^{-3}), manganese (24.7 mg dm^{-3}), zinc (3.39 mg dm^{-3}) and copper (1.23 mg dm^{-3}).

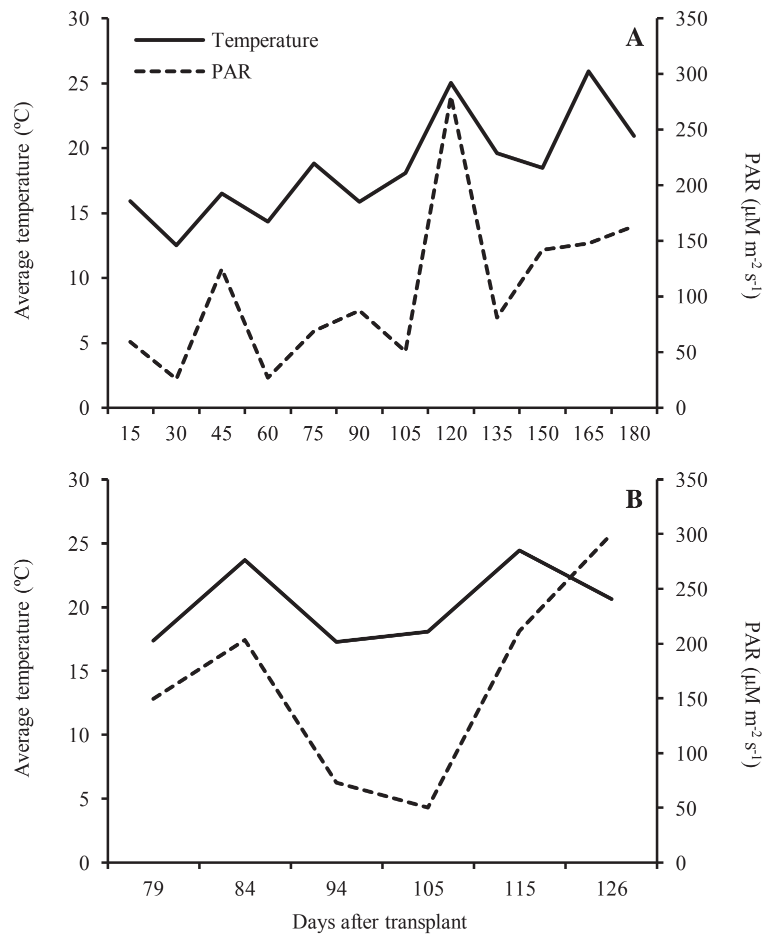

Figure 1. Average temperature and photosynthetically active radiation (PAR) recorded in the greenhouse during strawberry crop (A) and during harvest season (B). UPF, Passo Fundo, 2014.

2.4 AM root colonization

Root samples were washed thoroughly with running tap water to remove the adhered soil particles and cut into small pieces of about 1 cm according to Phillips & Hayman (1970). AM root colonization was quantified placing 1 cm segments onto a microscope slide and observed under microscope (TROUVELOT et al., 1986).

Accordingly, 100 intersections were quantified for each sample and the presence of any vesicles, arbuscules, or internal hyphae was assessed and percent root colonization was calculated from the number of colonized intersections out of total root intersections.

Before transplant, the roots of the plantlets present 5% AM root colonization.

2.5 Leaf appearance rate and phyllochron

Evaluations were determined by counting the number of leaves, twice a week, from the onset of leaf emission to the first fruit harvest. A new leaf was considered as emitted when it was visible with approximately 1 cm length.

The daily mean temperature (Dmt) was assessed according to the following equation, which calculates the arithmetic mean of temperatures recorded by the weather station (Watchdog) every hour:

$$Dmt = \frac{(t0+t1+t2\ldots+t23)}{24} \quad (^{\circ}C)$$

The daily thermal sum (Dts) was calculated according to Gilmore & Rogers (1958) and Arnold (1960):

$$Dts = Dmt - Bt \quad (^{\circ}C\ day^{-1})$$

Base temperature (Bt) is defined as the minimal temperature, below which there is no leaf appearance. The Bt for strawberry crop is considered 7 °C (RIEGER, 2007). The Dts was accumulated from transplantation, resulting in the accumulated thermal time (ATT):

$$ATT = \sum Dts \quad (^{\circ}C\ day^{-1})$$

Regression analysis was performed between the number of leaves in the main crown and accumulated temperature sum in each phenological stage. The angular coefficient of the linear regression is the LAR (leaves ºC day^{-1}) and the phyllochron (ºC day $leaf^{-1}$) was estimated by the inverse of the angular coefficient of the linear regression (KLEPPER et al., 1982; KIRBY, 1995; STRECK et al., 2007).

2.6 Growth and development

Strawberry plants were evaluated for growth parameter at blooming, fructification and at the end of cycle.

After removing the sand, root system was submerged in a clear graduated cylinder and the volume of water displaced was recorded as root volume (cm^3). Crown diameter (mm) was measured in all plants using a digital caliper (Vonder PDV 1500).

Plants were then divided into roots, crowns and leaves. Fresh biomass was measured by using a digital scale (Bel, capacity 2200 g). For dry biomass evaluations, shoot, roots and crown were placed in paper bags and dry in an oven at 65 °C to constant weight, then proceeding weighing, values expressed in g $plant^{-1}$.

2.7 Fruit yield

On the first week of September 2014 the plants started produce fruits until the third week of December.

For the variables related to the fruit yield, it was determined, monthly, in four plants of each experimental unit, the number and fresh mass (g $plant^{-1}$) of marketable fruit, according to the

classification adopted by Ceagesp (2002). Fresh mass of fruits was obtained by weighing in a digital scale. It is considered marketable fruits those with more than 6 g, devoid of injuries, diseases and deformations. The fruits were harvested when they had at least ¾ red.

2.8 Statistical analysis

Growth and development variables and AM root colonization were assessed for all treatments (inoculation x phenological stages), using split-plot analysis of variance (ANOVA). The effects of inoculation on yield were evaluated using one-way ANOVA with repeated measures (i.e. harvest season). Means comparisons were made using Tukey's test, differences at $p \leq 0.05$ were considered significant. Pearson correlation between variables analysed. Statistical analyses were performed using COSTAT (CoHort Software, 2003).

3 RESULTS

3.1 AM root colonization

AM root colonization varied among the treatments without interaction between inoculum and phenological stages (Table 1). All plants showed AM root colonization that was characterized by the presence of intraradical hyphae, arbuscules and vesicles. The control showed the lowest value since non-inoculation was applied. AM root colonization in non-inoculated plants in our study can be explained by the fact that the plantlets used came from a nursery with production on soil with possible presence of AM fungi. Before transplant, the roots

of the plantlets present 5% AM root colonization. However, no clear trend was observed in respect of AM root colonization. Overall, AM infection was higher for the treatments with inoculation, but the inoculation with only one species (Isolate 2) resulted in higher root colonization (Table 1).

Table 1. Arbuscular mycorrhizal (AM) fungi root colonization of strawberry plants (cv. Camarosa) with different inoculum evaluated during the crop season. UPF, Passo Fundo, 2014

Treatment	AM root colonization (%)
Inoculum	
Control	36.49 ± 31.43 b
Indigenous	46.70 ± 41.14 ab
C. etunicatum SCT101A	52.08 ± 40.44 ab
C. etunicatum MGR288A-1	56.98 ± 34.66 a
Phenological stages	
Blooming	9.40 ± 8.94 c
Fructification	50.94 ± 31.59 b
End of the cycle	83.85 ± 13.55 a
Mean	48.06
CV(%)	41.93

Means followed by the same letter are not significantly different according to Tukey's test (0.05). CV: coefficient of variation.

The extent of AM root colonization was modulated according to phenology, increasing from blooming to the end of the crop harvest (Table 1). At earlier stages of plant development, the rate of AM root colonization has been lower as colonization rate increase with plant age.

3.2 Leaf appearance rate and phyllochron

Phenological stages evaluated were vegetative, blooming and fructification, which lasted 31, 20 and 21 days, respectively. Thus, the early harvest occurred at 71 days after transplanting, when plants accumulated 612.31 ºC day^{-1} (Figure 2). During this period, the plants showed an average seven leaves and the interval between emissions of two leaves was in average of 10 days for all treatments.

The air temperature influenced the leaf appearance, represented by linearity and high coefficient of determination between leaf number and the accumulated thermal sum for all treatments (Figure 2).

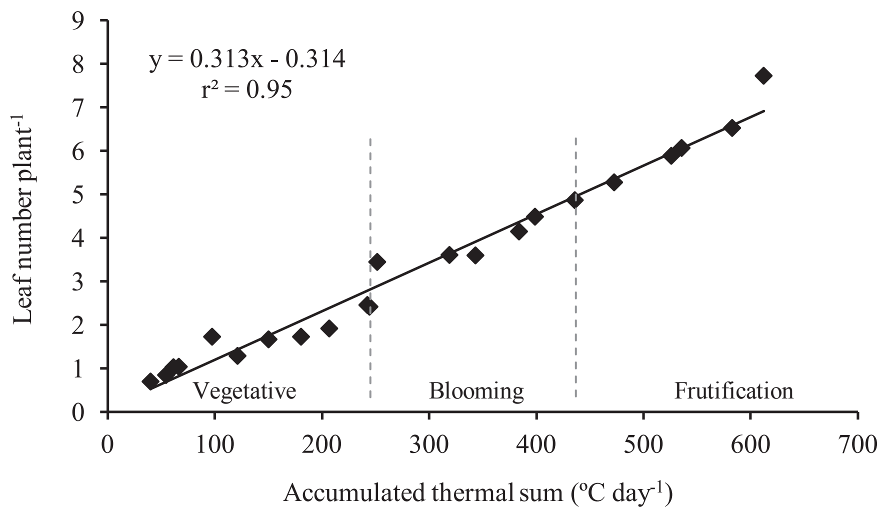

Figure 2. Relation between leaf numbers accumulated in the main crown and accumulated thermal sum in strawberry plants (cv. Camarosa) during growth season. UPF, Passo Fundo, 2014.

According to statistical analysis it was observed interaction for LAR between inoculum and phenological stages (Table 2). Among phenological stages LAR increased along crop development ($LAR_{vegetative} < LAR_{flowering} < LAR_{fructification}$) for plants that received AM inoculation, do not observed for control plants. While for inoculum, only at flowering was observed effect of AM inoculation for LAR (Table 2).

Table 2. Leaf appearance rate in strawberry plants (cv. Camarosa) inoculated with arbuscular mycorrhizal fungi along the phenological stages. UPF, Passo Fundo, 2014

Inoculum	Phenological stages		
	Vegetative	Blooming	Fructification
	-------------------------------- leaf °C day^{-1} --------------------------------		
Control	B 0.0074 ± 0.0033 a	A 0.0170 ± 0.0119 a	AB 0.0111 ± 0.0054 a
Native	B 0.0055 ± 0.0033 a	AB 0.0080 ± 0.0009 b	A 0.0142 ± 0.0038 a
C. etunicatum SCT101A	B 0.0090 ± 0.0015 a	B 0.0097 ± 0.0022 ab	A 0.0184 ± 0.0038 a
C. etunicatum MGR288A-1	A 0.0088 ± 0.0014 a	A 0.0100 ± 0.0008 ab	A 0.0153 ± 0.0017 a
Mean	0.0112		
CV(%)	29.16		

Means followed by the same lower case letters in the column and capital letters in the line do not differ significantly by the Tukey test ($p < 0.05$). ns: non-significative. CV: coefficient of variation.

Phyllochron values, obtain from regression equations, showed interaction between source of inoculum and phenological stages (Table 3). Effects of AM inoculation on phyllochron were observed only at vegetative and fructification stages (Table 3). In the vegetative stage, plants inoculated with native community required more accumulated thermal sum for the leaf emission. However, during fructification the same response was observed in plants that received no source of AM inoculum.

Table 3. Phyllochron of strawberry plants (cv. Camarosa) inoculated with arbuscular mycorrhizal fungi along phenological stages. UPF, Passo Fundo, 2014

Inoculum	Phenological stages		
	Vegetative	Blooming	Fructification
	------------------------------- °C day leaf^{-1} -------------------------------		
Control	A 173.07 ± 116.42 ab	A 89.51 ± 63.20 a	A 129.40 ± 110.17 b
Native	A 251.09 ± 152.62 a	B 125.65 ± 12.67 a	B 74.61 ± 20.46 a
C. etunicatum SCT101A	A 114.55 ± 22.31 b	A 107.60 ± 26.80 a	A 56.19 ± 12.10 a
C. etunicatum MGR288A-1	A 116.04 ± 20.86 b	A 100.07 ± 9.06 a	A 65.95 ± 7.33 a
Mean		116.98	
CV(%)		40.64	

Means followed by the same lower case letters in the column and capital letters in the line do not differ significantly by the Tukey test ($p < 0.05$). ns: non-significative. CV: coefficient of variation.

All the treatments with some source of AM inoculum reduced, in absolute numbers, the phyllochron. Nevertheless, this response was significant only for the plants inoculated with native community, which decreased by 50 and 70% on flowering and fructification, respectively, compared to the vegetative stage (Table 3).

3.3 Growth and development

Plant variables were not significantly different at inoculum treatments, however, increased during crop season (Table 4 and 5).

The plants do not respond to AM inoculation; however, growth parameters were positively correlated to AM root colonization percentage (Table 6). AM root colonization increased during crop season (Table 1) as well as plant growth parameters (Table 4 and 5).

Our results indicate that the percentage of AM root colonization leads to an increase in growth, without differences among the inoculum.

Table 4. Root volume and crown diameter of strawberry plants (cv. Camarosa) inoculated with arbuscular mycorrhizal fungi and during the crop season. UPF, Passo Fundo, 2014

Treatment	Root volume (cm^3)	Crown diameter (mm)
Inoculum		
Control	48.53 ± 28.24^{ns}	17.11 ± 5.80^{ns}
Indigenous	50.79 ± 29.29	17.43 ± 4.89
C. etunicatum SCT101A	49.69 ± 29.88	17.17 ± 4.66
C. etunicatum MGR288A-1	50.03 ± 30.00	18.44 ± 6.18
Phenological stages		
Blooming	15.38 ± 4.18 b	12.07 ± 1.00 c
Fructification	61.04 ± 20.85 a	16.82 ± 1,71 b
End of the season	72.86 ± 10.60 a	23.73 ± 3.11 a
Mean	49.76	17.54
C.V. (%)	24.01	12.23

Means followed by the same letter in the column are not significantly different according to Tukey's test ($p<0.05$). ns: non-significative. CV: coefficient of variation.

Table 5. Fresh and dry biomass of strawberry plants (cv. Camarosa) inoculated with arbuscular mycorrhizal fungi and during the growth season. UPF, Passo Fundo, 2014

Treatment	Fresh biomass (g plant^{-1})			Dry biomass (g plant^{-1})		
	Leaves	Root	Crown	Leaves	Root	Crown
Inoculum						
Control	93.20 ± 65.58^{ns}	47.60 ± 28.28^{ns}	8.21 ± 6.97^{ns}	25.29 ± 12.65^{ns}	10.99 ± 8.61^{ns}	2.34 ± 2.19^{ns}
Indigenous	91.63 ± 62.42	53.82 ± 31.79	8.82 ± 6.11	24.37 ± 18.05	13.11 ± 9.52	2.32 ± 1.65
C. etunicatum SCT101A	104.67 ± 66.52	55.43 ± 36.50	8.25 ± 4.90	28.73 ± 20.42	13.62 ± 11.59	2.26 ± 1.50
C. etunicatum MGR288A-1	89.37 ± 61.59	50.88 ± 28.42	9.69 ± 7.38	23.50 ± 16.32	12.03 ± 8.35	2.90 ± 2.30
Phenological stages						
Blooming	22.03 ± 4.66 c	16.64 ± 3.83 c	2.81 ± 0.64 c	4.16 ± 0.92 c	2.06 ± 0.60 b	0.68 ± 0.18 c
Fructification	107.47 ± 44.98 b	63.26 ± 26.25 b	7.67 ± 2.21 b	26.74 ± 6.70 b	18.32 ± 6.03 a	2.14 ± 0.84 b
End of the season	154.65 ± 35.88 a	75.89 ± 47.94 a	15.75 ± 5.01 a	45.53 ± 8.22 a	16.92 ± 4.29 a	4.54 ± 1.56 a
Mean	94.72	51.93	8.74	25.47	12.43	2.45
C.V. (%)	29.09	36.69	37.20	21.62	56.15	40.16

Means followed by the same letter in the column are not significantly different according to Tukey's test ($p<0.05$). ns: non-significative. CV: coefficient of variation.

Table 6. Correlation between growth attribute of strawberry plants and AM root colonization percentage, independent of AM inoculation (*n*=48, p<0.0001). UPF, Passo Fundo, 2014

Attribute	Coefficient of correlation (%)
Fresh biomass (g plant^{-1})	
Leaf	0.70***
Crown	0.71***
Root	0.69***
Dry biomass (g plant^{-1})	
Leaf	0.76***
Crown	0.69***
Root	0.58***
Root volume (cm^3)	0.71***
Crown diameter (mm)	0.72***

***: p<0.0001.

3.4 Fruit yield

Number of fruits and fruit size and fresh mass were similar for all AM fungi treatments and control (Table 7).

Fruit number, diameter, fresh mass and yield varied among the harvest period with lower values in November and December (Table 7). On the first week of September 2014 strawberry plants started produce fruits until December. However, in November and December the number of fruit harvested was really low due to high temperatures during October (Figure 1) that reduced fruit size and fresh mass, and also yields as a result of flower abortion.

Table 7. Yield components of strawberry plants inoculated with AM fungi and during growth season. UPF, Passo Fundo, 2014

Treatments	Yield components					
	Fruit plant^{-1}	Fruit diameter (mm)	Fruit (g plant^{-1})	Average fruit weight (g)	Marketable fruit	
					plant^{-1}	g plant^{-1}
Inoculum						
Control	3.50 ± 3.32^{ns}	27.20 ± 7.83^{ns}	47.47 ± 52.81^{ns}	13.13 ± 8.13^{ns}	1.78 ± 2.35^{ns}	30.91 ± 45.88^{ns}
Indigenous	3.71 ± 3.66	27.25 ± 5.71	51.93 ± 60.67	12.61 ± 4.94	2.19 ± 2.57	34.88 ± 45.54
C. etunicatum SCT101A	3.53 ± 3.04	25.32 ± 6.72	47.04 ± 53.90	11.31 ± 6.42	2.09 ± 2.62	34.44 ± 47.25
C. etunicatum MGR288A-1	2.59 ± 2.13	28.27 ± 5.70	34.06 ± 31.12	12.56 ± 5.70	1.55 ± 1.56	22.41 ± 25.03
Harvest period						
September	5.54 ± 1.87 a	24.19 ± 1.91 b	56.46 ± 26.26 b	9.92 ± 2.21 bc	2.30 ± 1.51 b	28.55 ± 23.04 b
October	5.95 ± 2.77 a	32.08 ± 4.09 a	03.68 ± 51.10 a	18.21 ± 4.36 a	4.65 ± 2.10 a	82.25 ± 43.49 a
November	0.50 ± 0.25 b	31.04 ± 7.03 a	7.75 ± 5.73 c	15.10 ± 4.57 ab	0.33 ± 0.26 c	6.56 ± 5.24 b
December	1.00 ± 0.78 b	20.67 ± 3.49 b	6.62 ± 6.56 c	6.18 ± 1.98 c	0.06 ± 0.17 c	0.54 ± 1.97 b
Mean	3.33	27.01	45.13	12.40	1.90	30.66
CV (%)	48.43	17.54	63.69	36.07	67.90	83.17

Means followed by the same letter in the column are not significantly different according to Tukey's test ($p<0.05$). ns: non-significative. CV: coefficient of variation.

AM root colonization failed to produce any significant effect according to correlation (Table 6).

Table 8. Correlation between average yield components of strawberry plants and AM root colonization percentage, independent of AM inoculation (*n*=16). UPF, Passo Fundo, 2014

Components	Coefficient of correlation (%)
Fruit plant^{-1}	-0,27^{ns}
Fruit diameter (mm)	0,28^{ns}
g fruit plant^{-1}	0,35^{ns}
Average fruit weight (g)	0,12^{ns}
Marketable fruit plant^{-1}	-0,27^{ns}
Marketable fruits (g plant^{-1})	-0,34^{ns}

ns: non-significative.

4 DISCUSSION

4.1 AM root colonization

The results showed that the AM root colonization rates were in the same range as previous studies, such as 25 to 75% (CHAVÉZ & FERRERA-CERRATO, 1990), 55.4 to 70.8% when inoculate with *Glomus* species (NORMAN et al., 1996).

There was an interspecific variability in relation to the AM fungi capacity to colonize the strawberry roots without interference of the phenological stages. In other words, there is specificity of AM specie and plant genotype.

The percentage of root colonization during the reproductive stages, regardless of the inoculation, was higher than that reported for strawberry after blooming (LINGUA et al., 2013). In this respect, fruit production is a major sink for carbon and plants reduced

assimilate supply of roots and, consequently, decrease AM colonization (SCHAARSCHMIDT et al., 2007).

4.2 Leaf appearance rate and phyllochron

The hypothesis was that applying AM fungi in strawberry could anticipate the flower and fruit production, reducing the period of vegetative growth. However, both inoculation with native community and a single species influence leaf appearance rate and phyllochron with different responses according to the plant phenological stage.

Inoculation with AM fungi in strawberry plants (cv. Camarosa) affects the LAR and phyllochron with different responses depending on the development stage. Nevertheless, the presence of the AM inoculum during transplanting do not resulted in precocity of strawberry, since plants from all treatments reached blooming, fructification and harvest at the same period. However, one of the effects of using AM fungi includes blooming and early fructification (BAREA et al., 1993). Phenology in tomato showed to be influenced by the presence of mycorrhiza, anticipating the blooming and fruit formation (SALVIOLI et al., 2012).

The interval between emissions of two leaves was in average of 10 days, regardless the treatment. Confirming the values found by Mendonça et al. (2012) that varied from 7 to 14 days in different cultivars grown in the same region in soilless crop system. The appearance of two successive leaves on strawberry take 8 to 12 days being the temperature the main factor that affects this physiological process (GALLETA & HIMELRICK, 1990). The linearity and high coefficient of determination (0.95) between leaf

number and accumulated thermal sum emphasize that air temperature was one of the decisive factor for the leaf emission in strawberry, agreeing with Pivetta et al. (2007) for tomato crop and Rosa et al. (2011) and Tazzo et al. (2015) for strawberry.

The interaction between inoculation and phenological stages demonstrated that LAR was influenced by AM fungi only in blooming with higher values for control plants in comparison with plants inoculated with native community (Table 2). In absolute number, the same trend was observed for phyllochron at the same period, in which control plants required lower accumulated thermal sum for leaf emission (Table 3). High phyllochron values in plants indicates that the rate of leaf appearance is lower as the plant requires greater number of degree-days between the appearance of two successive leaves (ROSA et al., 2011).

It was noted that the inoculated plants have different temperature requirements for the emission of leaves along its development. Among the treatments that received inoculation LAR increased during crop development (Table 2), consequently lower phyllochron values are observed in fructification (Table 3). It is important that the LAR is greater, consequently lower phyllochron, during the vegetative stage for the increased number of leaves before blooming occurs ensuring an increase in the number of fruits (ROSA et al., 2011).

The ability to emit leaves with lower accumulated thermal sum in inoculated plants during fructification may be result of high AM root colonization. Major number of leaves provides higher photosynthetic capacity and consequently greater accumulation of

assimilates that can be reversed to fruit production. However, some authors argue that during reproductive phase the growth of vegetative organs compete for photoassimilates with reserve organs such as fruits, and thus the emission of new leaves may be affected by plant isometrics relationships (SKINNER & NELSON, 1995; McCONNAUGHAY & COLEMAN, 1999). But, more studies should be done to undo this setback, since the main variables that control phenology of plants are the date of sowing/transplanting, day length, temperature, humidity, genetic component and plant management (DIAS et al., 2009).

4.3 Growth and development

The results are opposed to the hypothesis that different types of AM inoculum showed different responses to the growth and development of strawberry, based on distinct authors (KHANIZADEH et al., 1995, MARK & CASSELLS, 1996, MURPHY et al., 2000). Different AM-fungal species and even distinct geographic isolates of the same species can affect plant development differently (WILSON, 1988; BETHLENFALVAY et al., 1989). AM fungi can either enhance or reduce the growth of strawberry shoots and roots (TAYLOR & HARRIER, 2001; VESTBERG et al., 2004). Indigenous isolates are not necessarily highly effective in terms of mycorrhizal growth response (KOOMEN et al., 1987).

The increase of AM root colonization followed by the increased in plant growth suggest a positive effect of these fungi as the symbiosis is established. These results indicate that the inoculation

with these fungi must be performed during transplant, even in the nursery during plantlets production.

Root volume and biomass were positively correlated with AM root colonization, in agreement with previous studies that linked the level of mycorrhizal colonization to the level of roots system enhancement (ATKINSON et al., 1994).

4.4 Fruit yield

No response was observed for fruit yield in strawberry plants inoculated with AM fungi (Table 7). On other cases, the increases in the numbers of flowers and fruit in inoculated strawberry plants have been reported (ROBERTSON et al., 1988). And also, similar results in g per fruit for the strawberry cultivars inoculated with AM fungi (CEKIC & YILMAZ, 2011), diverging from our results. Fungal inoculation of strawberry plants might produce differing responses in terms of plant growth and fruit production. The application of commercially available or native AM fungi inoculants to strawberry plants resulted in higher root colonization levels, leaf area but did not result in increased yield (GARLAND et al., 2011). No differences in marketable yield were detected between the inoculated and non-inoculated plants of strawberry cultivar (BULL et al., 2005). Mycorrhization through commercial product increased fruit weight in different strawberry cultivars but had no effects on yield (FRAC et al., 2009). Increased in strawberry fruit yield was already reported by Douds et al. (2008) followed by AM fungi inoculation using on farm inoculum, as well as recently by Bona et al., 2015.

High temperature can reduce pollen quality and result in pollination failure (WILCOCK & NEILAND, 2002). Strawberry pollen germination and pollen tube growth failure at above 26°C lead to a decline in flower to fruit conversion (LEDESMA & SUGIYAMA, 2005; KARAPATZAK et al., 2012), consequently decrease yield.

Average total yield (180.50 g $plant^{-1}$), regardless of the presence or absence of AM inoculation, were lower than those recorded for the same cultivar (cv. Camarosa) in a soilless growth system (225 g $plant^{-1}$, CECATTO et al., 2013). However, those authors harvest the plants from September to January, while in our study harvest was performed from September to December. In addition, the lower values for yield are related to flower abortion in November and December due to high temperatures (Figure 1).

It is important to point out that in our study the average of photosynthetically active radiation (PAR) inside the greenhouse was 200 μmol m^{-2} s^{-1}, below the ideal range for strawberry flower and fruit production of 400 to 450 μmol m^{-2} s^{-1} (KIRSCHBAUM, 1998), values only recorded around of 10-15 h, over the crop season. It is known that PAR radiation can increase the production of photoassimilates and their availability for growth and fruit production (ANDRIOLO, 2000).

AM fungi have previously been shown to alter plant reproduction physiology, inducing earlier blooming, prolonged blooming period, and increased the number of flower buds, inflorescences, and fruits (LU & KOIDE, 1994; HARRIER & WATSON, 2003).

The inoculation with native or isolates of AM fungal did not influence growth, development and yield on strawberry plants (cv. Camarosa). However, the level of AM root colonization varied among the treatments with positive correlation with growth parameters. The inoculation of various AM fungi isolated from different soils and sources may vary in term of effectiveness and their infectivity (JAKOBSEN & NIELSEN, 1983).

The variability in response of strawberry to inoculation with AM fungi may be due to several factors such as cultivar and/or AM fungi specific. Effects have been noted for strawberry with interaction between cultivar and AM fungus (CHAVEZ & FERRERA-CERRALO, 1990; STEWART et al., 2005), and diversity of AM fungi in the inoculum can contribute to the response (KOOMEN et al., 1987). In addition, inoculation at the time of transplanting may be ineffective. Thereby, inoculation prior to transplant may achieve positive response on growth and yield using a taxonomically diverse inoculum.

The efficiency of mycorrhizal symbiosis in horticulture plants depends on climatic factors, soil and/or substrate, agronomic practices, plant genotype and AM fungi species (ELBON & WHALEN, 2015). One of the factors that may have influenced these results was the moment of the inoculation, during the transplanting. In horticulture, researches related to AM inoculation time is relevant since it is possible introduce AM fungus during plantlets production what increases the chances of success of the symbiosis (DOUDS et al., 2008). Even without positive responses, further experiments should be carried out testing the effects of mycorrhizal inoculation on

different cultivars and sources of AM fungi. Since AM fungi can improve plant growth by impacting phytohormone balance that stimulates plant development (SMITH & READ, 2008) and positive responses related to growth and yield were obtained on inoculated strawberry plants (DOUDS et al., 2008).

5 CONCLUSION

Inoculation with arbuscular mycorrhizal fungi in strawberry plants (cv. Camarosa) influences the leaf appearance rate and phyllochron during the development stages. However, the present study was inconclusive regarding the benefit of AM on growth and yield parameters. Thus, strawberry plants inoculated with AM fungi under soilless crop system should remain for a longer period in cultivation so that the benefits of the symbiosis can be expressed.

CHAPTER III

ACCUMULATION OF SECONDARY METHABOLISM COMPOUDS IN STRAWBERRY ASSOCIATED WITH ARBUSCULAR MYCORRHIZAL FUNGI

ANA CLÁUDIA PEDERSEN

ABSTRACT - There is increasing interest in the quality of crops because of the implications concerning health, economic revenue, and food quality. Strawberries contain a wide range of nutrients and bioactive compounds. Arbuscular mycorrhizal (AM) fungi can interact with plants and has a positive effect on various plant growth parameters but can also affect the quality of crops products. The objective of this study was to test if AM fungi inoculation influences fruit quality parameters and in strawberry (cv. Camarosa) in soilless system. The treatments consisted in AM fungi inoculation: i) control; ii) native AM fungi community; iii) isolate 1 (*Claroideoglomus etunicatum* SCT101A); and iv) isolate 2 (*C. etunicatum* MGR288A-1). The treatments were distributed in a randomized block design with four replications and four plants per plot, considering all plants for evaluation. The effects of inoculation were evaluated using one-way analysis of variance (ANOVA) with repeated measures. Strawberry plants inoculated with mycorrhizal fungi exceed the percentage of root colonization of the control plants. Interaction between harvest season and AM inoculation was observed only for total phenolic, anthocyanins and flavonoids content, and antioxidant activity. The introduction of AM fungi into strawberry plants, during transplant in

soilless growth system, increases the fruit quality parameters such as content of total phenolic, anthocyanins and flavonols but also the antioxidant activity.

Key words: anthocyanins, antioxidant activity, flavonoids, *Fragaria* x *ananassa* Duch., polyphenols

ACÚMULO DE COMPONENTES DO METABOLISMO SECUNDÁRIO EM MORANGUEIRO ASSOCIADO COM FUNGOS MICORRÍZICOS ARBUSCULARES

RESUMO - Existe um interesse crescente na qualidade das culturas por causa das implicações na saúde humana, rentabilidade e qualidade dos alimentos. Morangos contem ampla variedade de nutrientes e compostos bioativos. Fungos micorrízicos arbusculares (MA) podem interagir com plantas com efeito positivo sobre os parâmetros de crescimento de plantas, mas também podem afetar a qualidade dos produtos das culturas. O objetivo desse estudo foi testar se a inoculação com fungos MA influencia nos parâmetros de qualidade do morangueiro (cv. Camarosa) no sistema de cultivo sem solo. Os tratamentos consistiram de fontes de inóculo micorrízico: i) controle; ii) comunidade nativa de fungos MA; iii) isolado 1 (*Claroideoglomus etunicatum* SCT101A) e iv) isolado 2 (*C. etunicatum* MGR288A-1). Os tratamentos foram distribuídos em blocos ao acaso com quatro repetições e quatro plantas por parcela, considerando todas as plantas para avaliação. O efeito da inoculação foi avaliado usando análise de variância (ANOVA) com medidas repetidas no tempo.

Plantas de morangueiro inoculadas com fungos MA excedem a porcentagem de colonização micorrízica das plantas de controle. Observou-se interação entre época de colheita e inoculação micorrízica apenas para o conteúdo de fenólicos totais, antocianinas e flavonóides e a atividade antioxidante. A introdução de fungos MA em plantas de morangueiro, durante o transplante no sistema de produção fora do solo, aumenta os parâmetros de qualidade dos frutos.

Palavras-chave: antocianinas, atividade antioxidante, flavonoids, *Fragaria* x *ananassa* Duch., polifenóis.

1 INTRODUCTION

Researchers in horticulture has been adding quality parameters in addition to the traditionally studied as yield. The focus of several studies is due to implications on prevention of chronic diseases (HANNUM, 2004). Strawberry (*Fragaria* x *ananassa* Duch.) fruits are rich in bioactive phenolic compounds including flavonoids, phenolic acids and anthocyanins (TULIPANI et al., 2008; WANG & MILLNER, 2009) with high antioxidant activity (CHU et al., 2002; HALVORSEN et al., 2002; MEYERS et al. 2003). The chemical composition of strawberry varies with genotype (ANTTONEN et al., 2006; CAPOCASA et al., 2008; CORDENUNSI et al., 2002; OLSSON et al., 2004; WANG et al., 2002; WANG & ZHENG, 2001), but is also affected by factors such as agricultural practice, environment, and maturity (PINELI et al., 2011)

Crop management practices aimed at increasing yield and food quality while lowering environment impacts are a major current requirement (ANTUNES et al., 2012). A strategy to achieve this is the use of beneficial soil microorganisms such as arbuscular mycorrhizal (AM) fungi, which are able to colonize plant roots and establish a mutualistic symbiosis with most of vascular plants (SMITH & READ, 2008). Strawberry is highly responsive to AM fungi (BORKOWSKA, 2002; CASTELLANOS-MORALES et al., 2010; DOUDS et al., 2008). It has been shown that some AM fungal isolates positively affect its growth and development (VESTBERG et al., 2004; MALUSA et al., 2007) and yield (DOUDS et al., 2008).

Beneficial soil microorganism, like AM fungi, can influence the plant secondary metabolic pathway (ZENG et al., 2013). Plants inoculated with AM fungi have been shown to contain greater amounts of these metabolic compounds that improve the quality of a variety of crops such as *Olea europaea* L. (olive trees) (GANZ et al., 2002), *Ocimum basilicum* L. (sweet basil) (SCAGEL & LEE, 2012; TOUSSAINT et al., 2007), *Lactuca sativa* L. (lettuce) (BASLAM et al., 2011a,b), *Cynara cardunculus* var. *scolymus* F. (artichoke) (CECCARELLI et al., 2010), *Solanum lycopersicum* L. (tomato) (GIOVANNETTI et al., 2012; COPETTA et al., 2011).

The use of mycorrhizal technology to induce the accumulation of secondary metabolites in plants is advantageous because are important for human health with antioxidant, anti-inflammatory, anti-bacterial, antiproliferative and antiviral properties (ROSS & KASUM, 2002). These compounds offer protection against development of cancers, cardiovascular diseases, diabetes,

osteoporosis and neurodegenerative diseases (GRAF et al., 2005; ARTS & HOLLMAN, 2005). But also, because it contributes to the development of sustainable agriculture, reducing the application of chemicals fertilizers (PEDONE-BONFIM et al., 2013) as AM fungi increase the absorption of nutrients (TOUSSAINT, 2008). In addition, phenolic compounds act as defensive compounds against environmental stresses, herbivores and pathogens (LATTANZIO et al., 2006; STEINKELLNER et al., 2007, CHALKER-SCOTT, 1999) what enables reduction on pesticides inputs.

Studies have shown that AM fungi may be a potential strategy to increase secondary metabolites in strawberry (CASTELLANOS-MORALES et al., 2010; LINGUA et al., 2013). However, these studies focus on only one AM fungi genera (*Glomus*), day-neutral cultivars, and consortium with plant growth promoting bacteria and different fertilization. Quantitative and qualitative changes in secondary metabolite composition of plants colonized by AM fungi could have significant effects on quality of strawberry fruits. In addition, improve product quality is an additional goal in agriculture due to attention, by the consumers, to aspects of food quality regarding both health and environment. Thereby, it was hypothesized that using an indigenous community of AM fungi, in a strawberry soilless crop system, can improve fruit quality. The objectives of this study were to test if inoculation with AM could increase quality parameters of strawberry fruits growing under soilless system conditions.

2 MATERIAL AND METHODS

2.1 Plant material and mycorrhizal inoculum

From June to December 2014, an experiment was carried out in a greenhouse in Passo Fundo/RS/BR (28º15'41" S, 52º24'45" W, elevation 709 m). It was used bare roots plantlets of strawberry (cv. Camarosa) from Agrícola LLahuen nursery, Chile (33º50'30.19" S, 70º40'05.04" W), provided by Strawberry Breeding Program at the University of California. The plantlets production is performed at open field after soil sterilization. After two months the commercial plantlets are harvest mechanically and prepared by washing and selected according to the crown diameter. The plantlets are packed leafless and sent to Brazil in containers and delivered after 10-12 weeks.

Field soil from a strawberry crop (cv. Camarosa) at Universidade de Passo Fundo (28º15'41"S, 52º24'45"W, 709 m) was collected in January 2014 to use as an inoculum containing a native community. Others soils from a strawberry crop were tested. As criteria for choosing the soil used as inoculum was sporulation in the trap culture and mycorrhizal inoculum potential.

Trap cultures were established to trigger sporulation of native AM fungi species. Traps were set up and let to grow for 18 weeks under greenhouse conditions at University of Passo Fundo. Traps were established by placing 200 g of soil sample, containing strawberry root pieces, as layers between two layers of sterilized sand (121 ºC, 1h, two cycles with 24h interval), in 1.0 kg plastic pots. Approximately 60 seeds of Sorghum sudanense, as a host plant, were sown at 2 cm depth in each pot and covered with sterilized sand. Pots

were irrigated daily and no fertilization was added during the growth period. After 18 weeks of growth, 50 g soil was sampled from each pot for AM fungi spore extraction and identification. Arbuscular mycorrhizal fungi spores were extracted by the wet sieving method described by Gerdemann and Nicolson (1963) followed by sucrose gradient centrifugation (20% and 60%). AM fungi species were identified, at University of Blumenau-Brazil, based on subcellular structures of asexual spores following description of living reference cultures (http://invam.caf.wvu.edu) and their classification according to Redecker et al. (2013). Also was used two AM fungi isolated specie as inoculum (C. etunicatum SCT101A and C. etunicatum MGR288A-1), originally isolated from Brazil, obtained from Coleção Internacional de Cultura de Glomeromycota (CICG; http://www.furb.br/cicg).

Annual average of accumulated hours of chill is 422 hours, with lower or equal temperature to 7.0ºC ranging from 214 to 554 hours (CUNHA, 2003).

2.2 Treatments and experimental design

The treatments consisted in inoculum and phenological stages. Inoculum: (1) control; (2) indigenous community consisting of *Acaulospora mellea*, *A. morrowiae*, *Cetraspora pellucida*, *Claroideoglomus etunicatum*, *Glomus* sp. and *Septoglomus viscosum*; (3) Isolate 1 - *C. etunicatum* SCT101A; and (4) Isolate 2 - *C. etunicatum* MGR288A-1. Phenological stages: blooming; fructification; and, end of the cycle.

The treatments were distributed in a split-plot design with four replicates. In the main plot was tested the type of inoculum and in the split-plot was tested the phonological stages. It was transplanted four plants per plot, all plants were evaluated. The plant population was 16 plants m^{-2}.

2.3 Experiment setup

Bare root plantlets of cv. Camarosa, were transplanted in white low-density polyethylene bags (150 μm thickness) tubing shaped (0.5 m length and 0.3 m width), placed on wood stand. The bags were filled with 20 dm^3 of autoclaved sand (121 ºC, 1h, two cycles with 24h interval) and four plantlets were transplanted per bag. Transplant was done on June 17th, 2014.

A drip-irrigation system was placed into the bags near each plant. Irrigation pulses with duration of 5 min were applied (at a volume of 0.35 L per pulse per plant) every day. The nutrient solution recommended by Calvete et al. (2007) was used for fertirrigation, once a week. The pH and electrical conductivity (EC) of the fertilizer solution and the solution drained by the bags was monitored. The fertilizer solution pH and EC range from 5.5 to 6.7 and 1.15 to 2.15 dS m^{-1}, respectively. The drain solution pH did not vary and EC range from 0.1 to 1.7 dS m^{-1}, among the treatments.

Temperature and photosynthetically active radiation (Figure 1) were measured inside the greenhouse using a weather Station (WatchDog), during the experiment.

Autoclaved sand were analysed for chemical characteristics (TEDESCO et al., 1995). The chemical analysis of the

sand showed the follow values for cation exchange capacity (7.8 $cmol_c\ dm^{-3}$), pH (5.7), phosphorous (9.9 $mg\ dm^{-3}$), potassium (53 $mg\ dm^{-3}$), calcium (2.7 $cmol_c\ dm^{-3}$), magnesium (1.1 $cmol_c\ dm^{-3}$), sulfur (5 $mg\ dm^{-3}$), boron (0.1 $mg\ dm^{-3}$), manganese (24.7 $mg\ dm^{-3}$), zinc (3.39 $mg\ dm^{-3}$) and copper (1.23 $mg\ dm^{-3}$).

2.4 AM root colonization

Root samples were washed thoroughly with running tap water to remove the adhered soil particles and cut into small pieces of about 1 cm according to Phillips & Hayman (1970). AM root colonization was quantified placing 1 cm segments onto a microscope slide and observed under microscope (TROUVELOT et al., 1986). Accordingly, 100 intersections were quantified for each sample and the presence of any vesicles, arbuscules, or internal hyphae was assessed and percent root colonization was calculated from the number of colonized intersections out of total root intersections.

Before transplant, the roots of the plantlets present 5% AM root colonization.

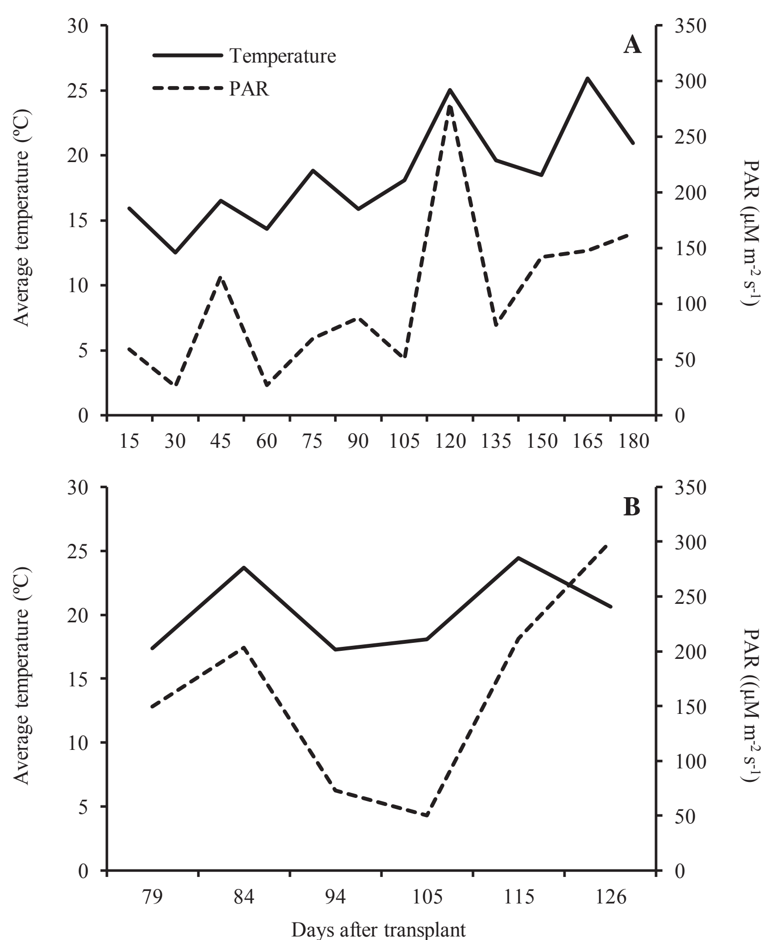

Figure 1. Average temperature and photosynthetically active radiation (PAR) recorded in the greenhouse during strawberry crop (A) and during harvest season (B). UPF, Passo Fundo, 2014.

2.5 Harvest and fruit evaluation

Harvest season happened from 79 to 126 days after transplant (DAT). Fruit quality was evaluated considering flavor, visual aspects and functional compounds (secondary metabolites).

To quantify variables that represent the flavor of fresh fruits total soluble solids (TSS) (Brixº) and titratable acidity (TA)

were analysed and TSS/TA calculated. The TSS content was obtained from five fresh fruit from each plot using refractometer. To determine TA, 10 g fruits of each sample was diluted in about 90 mL of distilled water and mixed with 0.3 mL of phenolphthalein solution. The titration was carried out with sodium hydroxide solution 0.1 M under constant stirring, until the pink color continuing for 30 seconds.

The transverse diameter of fruit was measured with a digital caliper.

For functional compounds were determined secondary metabolites such as total anthocyanins, flavonoids and phenolic content in addition to ferric reducing activity power. Fresh strawberry fruits were harvest, every 10 days, during harvest period. After harvest fruits were storage in plastic bags and frozen (-20 ºC) until analysis.

The extraction procedure was carried out according to Revilla et al. (1998). Fifty grams of frozen fruits were mixed with 50 mL of ethanol 70% pH 1.0 and soniphication at room temperature for 25 minutes. After extraction stage, samples were filtered and storage at -20ºC until analysis. All extractions were made in triplicates.

Total phenolic content was determined by FolinCiocalteu method according Singleton et al. (1999). Diluted extract (125 µL) were mixed with 500 µL of distilled water and 125 µL of Folin-Ciocalteu's phenol reagent. After incubated for 6 min sodium carbonate (7%) was added, and volume adjusted to 3 mL with distilled water. The mixture was incubated for 90 min at room temperature in the dark before absorption was measured at 760 nm (PerkinElmer Lambda 20 spectrophotometer, Perkin Elmer), using a linear calibration curve of gallic acid (0 - 680 mg L^{-1}) to calculate the total

phenol content. Total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per 100 g of fresh weight (mg of GAE/100 g of FW).

Anthocyanin content was determined by pH differential method described by Lee et al. (2005) and Giusti & Wrolstad (2001). Briefly, 500 µL of each sample were diluted with 2,0 mL aqueous pH 1.0 and 4.5 buffers and absorbance (A) measurements were taken at the 510 nm and 700 nm. Results were expressed as milligrams of cyanidine-3-glucoside per 100 g of fresh fruit weight, using the formula: $A = (A_{510} - A_{700})pH_{1.0} - (A_{510} - A_{700})pH_{4.5}$, with a molar extinction coefficient of cyanidine-3-glucoside of 29,600.

The total flavonoids content was determined as described by Miliauskas et al. (2004). Extract aliquots of 10 mL were diluted with 1 mL of aluminum chloride and volume adjusted to 25 mL with ethanol 70% pH 1.0. The absorbance measurements were taken at the 415 nm. The flavonoids content was determined by a rutin standard curve (0 – 5000 mg L^{-1}) and expressed as milligrams of rutin per liter of extract (mg rutin L^{-1}).

The antioxidant activity was quantified by ferric reducing activity power (FRAP) according to Zhu et al. (2002). Briefly, 250 µL of the sample was mixed with 1,25 mL of phosphate buffer (pH 7.0) and 1,25 mL of potassium ferricyanide (1%). The mixture was homogenized and incubated at 50°C for 20 min and subsequently on ice for 10 min. After, 1,25 mL of trichloroacetic acid (10%) was added and the samples were centrifuged at 3000 rpm for 10 min. The supernatant was mixed with 500 µL of ferric chloride hexahydrate (0.1 %). The samples were vortexed and absorbance was measured at

700 nm. The reagent blank was prepared by replacing the sample with ethanol 70% pH 1.0. Gallic acid was used as the standard antioxidant. The antioxidant capacity was expressed as gallic acid equivalent reducing power (mg GAE/100 g fruit weight).

2.6 Statistical analysis

Statistical analyses were performed using COSTAT (CoHort Software, 2003). The effects of inoculation were evaluated using one-way analysis of variance (ANOVA) with repeated measures. Means comparisons were made using Tukey's test, differences at $p \leq 0.05$ were considered significant. The results are presented as means of three replicates.

3 RESULTS

3.1 AM root colonization

Non-significative interaction was found for AM root colonization. However, significative differences were obtained for AM inoculation ($p=0.05$) and phonological stages ($p=0.00$) (Table1).

Plants from all treatments were colonized by AM fungi (Table 1). The control showed the lowest value considering that non-inoculation was applied. AM root colonization in non-inoculated plants in our study can be explained by the fact that the plantlets used came from a nursery with production on soil. Before transplant, the roots of the plantlets present 5% AM root colonization. However, no clear trend was observed among the inoculum in respect of AM root colonization. Overall, AM infection was higher for the treatments with

inoculation. Among the inoculated plants inoculation with only one species (Isolate 2) result in higher root colonization (Table 1).

The extent of AM root colonization was modulated according to phenology, increasing from blooming to the end of the cycle (Table 1).

Table 1. Arbuscular mycorrhizal (AM) fungi root colonization of strawberry plants with different inoculum along the phenological stages. UPF, Passo Fundo, 2014

Treatment	AM root colonization (%)
Inoculum	
Control	36.49 ± 31.43 b
Indigenous	46.70 ± 41.14 ab
C. etunicatum SCT101A	52.08 ± 40.44 ab
C. etunicatum MGR288A-1	56.98 ± 34.66 a
Phenological stages	
Blooming	9.40 ± 8.94 c
Fructification	50.94 ± 31.59 b
End of the cycle	83.85 ± 13.55 a
Mean	48.06
CV(%)	41.93

Means followed by the same letter are not significantly different according to Tukey's test (0.05). CV: coefficient of variation.

3.2 Fruit weight, diameter and flavour

On the first week of September 2014 (79 DAT) strawberry plants started produce fruits until December. However, in November there was no fruit harvested due to high temperatures during October (Figure 1 - beginning at 105 days after transplant) and in December the number of fruits was not enough to carry on the analysis.

AM inoculation failed to produce any significant effect on fruit weight, diameter and TSS/TA, which varied during harvest

season (Table 2). Higher values for fruit weight were observed at the end of the harvest season. It is also noted that the diameter growth did not follow the development of the fruit (g fruit^{-1}) in all harvest dates, only at the end of these, as well as the fruit flavor (TSS/TA).

Table 2. Fruit weight, fruit diameter and sugar and acidity ratio (TSS/TA) of strawberry fruits during harvest season. UPF, Passo Fundo, 2014

Treatment	Fruit weight (g fruit^{-1})	Diameter (mm)	TSS/TA
Harvest season (DAT)			
79	5.68 ± 7.04 b	1.15 ± 1.53 b	4.21 ± 4.98 b
84	10.93 ± 3.92 b	2.89 ± 0.30 a	8.77 ± 4.75 a
94	10.99 ± 3.87 b	2.47 ± 0.34 a	6.18 ± 2.32 ab
105	8.62 ± 5.03 b	1.00 ± 2.04 b	-
115	20.82 ± 6.38 a	3.24 ± 0.49 a	7.54 ± 4.09 ab
126	18.37 ± 5.18 a	3.61 ± 1.43 a	8.12 ± 3.38 ab
Inoculum			
Control	13.72 ± 6.46^{ns}	2.51 ± 1.24^{ns}	8.38 ± 5.07^{ns}
Indigenous	10.85 ± 7.68	2.08 ± 1.29	5.59 ± 4.08
C. etunicatum SCT101A	13.83 ± 6.00	2.24 ± 1.37	7.38 ± 3.06
C. etunicatum MGR288A-1	11.88 ± 9.36	2.45 ± 1.91	5.59 ± 4.34
Mean	12.56	23.22	6.96
CV(%)	39.19	40.12	56.55

Means followed by the same letter in the column are not significantly different according to Tukey's test (0.05). ns: non-significant; CV: coefficient of variation; DAT: days after transplant, TSS: total soluble solids; TA: titratable acidity.

3.3 Total phenolic, anthocyanins and flavonols content and antioxidant activity

Significant interaction was observed between AM inoculum during harvest season in total phenolic (TPC, $F=9.39$, $p=0.00$), anthocyanins ($F=42.72$, $p=0.000$) and flavonols content ($F=42.72$, $p=0.00$) and antioxidant activity ($F=34.56$, $p=0.00$) (Figure

2 and 3). The benefit of inoculation in the production of secondary metabolites and antioxidant activity occurred in almost all harvest. It is noticed that the native community and the *C. etunicatum* MGR288A-1 contributed to higher values in total phenolic compounds, flavonoids and anthocyanins. There was greater influence of both isolates for antioxidant activity (Figure 2 and 3).

The TPC ranged from 369.34 to 604.92 g GAE kg-1, both values in non-inoculated plants. Inoculation with AM fungi significantly modified the concentration of TPC only at 105 and 126 DAT (Figure 2). At these days, fruits inoculated with native AM fungi, *C. etunicatum* SCT101A and *C. etunicatum* MGR288A-1 had on average 51, 32 and 59% higher concentration of TPC than plant without inoculation, respectively.

Anthocyanin (Figure 3) and flavonoids (Figure 2) compounds had the same behaviour, ranging from 15.63 to 120.90 g cya-3-glu kg^{-1} and 2237.22 to 365.00 g rutin L^{-1}, respectively. Highest values were recorded for AM inoculum treatments except for fruits from the first bloom. For both parameters, lowest value corresponds to control plants at the last harvest and the highest to native inoculum treatment at the same period. At the last harvest, fruits from inoculated plants had anthocyanins content seven times higher and flavonoids five times higher than non-inoculated plants.

Regarding the content of secondary metabolites, the antioxidant activity, determined by FRAP, was higher for inoculated plants during the harvest season, than non-inoculated plant (Figure 3). FRAP showed wide variation from 141.54 to 385.10 g GAE kg^{-1}.

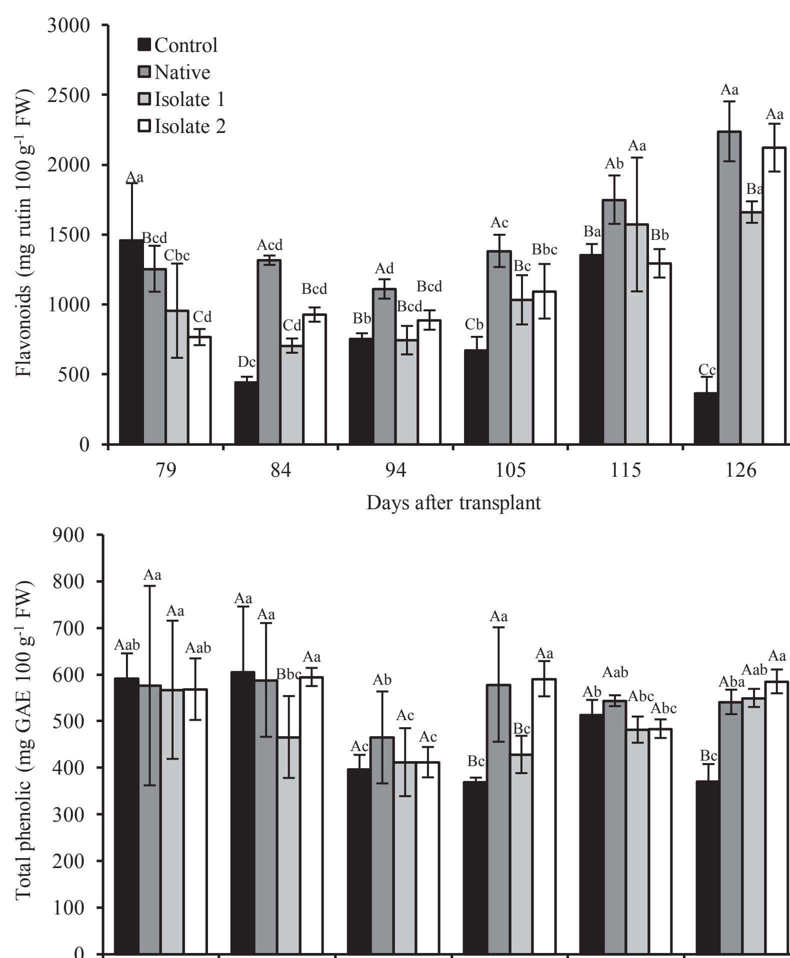

Figure 2. Flavonoids and total phenolic content on fruits from strawberry plants inoculated with arbuscular mycorrhizal fungi during harvest season. Means followed by the same capital letters among the treatments at the same day, and the same lower case letters for each treatment along the harvest season do not differs significantly by the Tukey's test ($p \leq 0.05$). UPF, Passo Fundo, 2014.

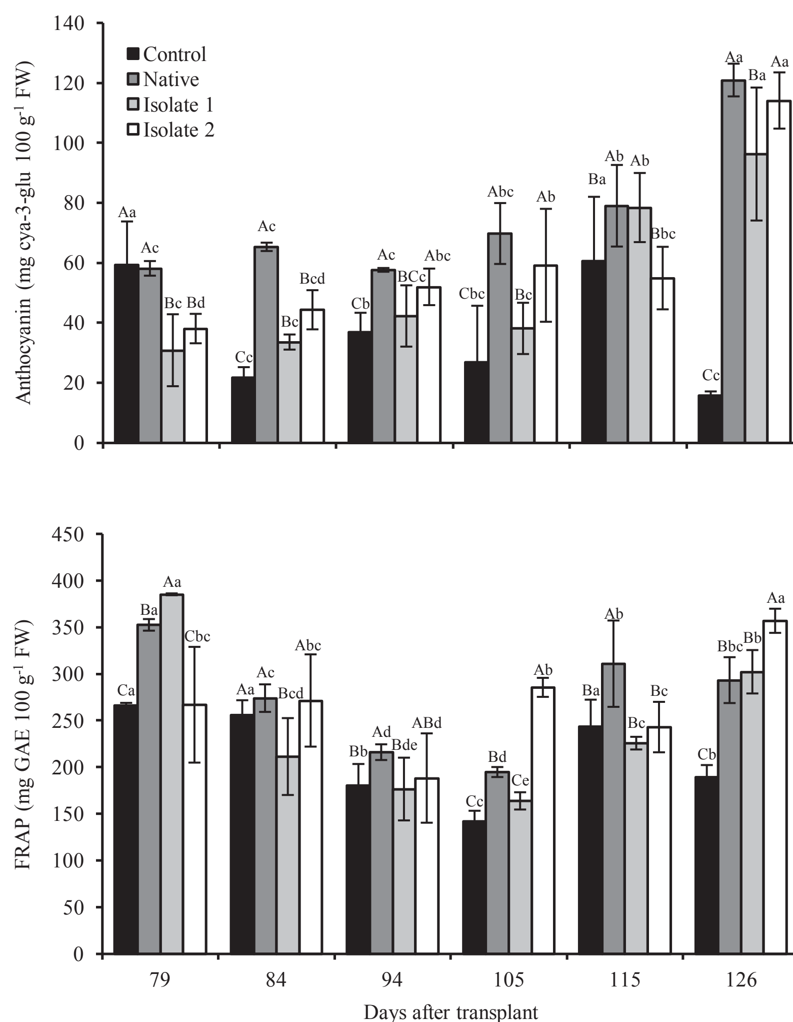

Figure 3. Anthocyanins content and antioxidative activity (FRAP) on fruits from strawberry plants inoculated with arbuscular mycorrhizal fungi during harvest season. Means followed by the same capital letters among the treatments at the same day, and the same lower case letters for each treatment along the harvest season do not differs significantly by the Tukey's test ($p \leq 0.05$). UPF, Passo Fundo, 2014.

4 DISCUSSIONS

The introduction of AM fungi into strawberry plants, during transplant in soilless growth system it is an important tool to increase the content of secondary metabolites. However, different AM inoculum (indigenous and isolates) varies in their efficacy to increase the synthesis of these compounds. We hypothesized that using an indigenous community of AM fungi, in a strawberry soilless crop system, could improve fruit quality, which was confirmed.

4.1 AM root colonization

AM fungi colonized plants from all treatments. AM root colonization in non-inoculated plants in our study can be explained by the fact that the plantlets used came from a nursery with production on soil. Overall, AM infection was higher for the treatments with inoculation, but the inoculation with only one species (*C. etunicatum* MGR288A-1) resulted in higher root colonization (Table 1).

Our results showed that the AM root colonization rates were in the same range as previous studies, such as 25 to 75% (CHAVÉZ & FERRERA-CERRATO, 1990), 55.4 to 70.8% when inoculate with *Glomus* species (NORMAN et al., 1996).

Despite of the same species (*C. etunicatum*) be present in all inoculum, the response related to AM root colonization was different. Different AM-fungal species and even distinct geographic isolates of the same species can affect plant development differently (WILSON, 1988; BETHLENFALVAY et al., 1989). Koomen et al. (1987) concluded that indigenous isolates are not necessarily highly effective in terms of mycorrhizal growth response. Root colonization

in strawberry under soilless growth system requires further investigation. Especially for indigenous communities, where researches are poorly explored.

At earlier stages of plant development, the rate of AM root colonization has been lower as colonization rate increase with plant age. This result contradicts the reduction of AM colonization on strawberry roots after blooming (LINGUA et al., 2013). In this respect, fruit production is a major sink for carbon and plants reduced assimilate supply of roots and, consequently, decrease AM colonization (SCHAARSCHMIDT et al., 2007).

4.2 Fruit weight, diameter and flavour

The absence of fruit in November and subsequent reduction of fruits in December can be a result of flower abortion due to high temperatures. High temperature can reduce pollen quality and result in pollination failure (WILCOCK & NEILAND, 2002). Strawberry pollen germination and pollen tube growth failure at above 26°C lead to a decline in flower to fruit conversion (LEDESMA & SUGIYAMA, 2005; KARAPATZAK et al., 2012), consequently decrease yield.

Fruit weight, diameter and flavour behavior were independent of AM inoculation, ranging along the harvest season (Table 2). Cekic & Yilmaz (2011) also found no effects of AM inoculation on fruit size, however, TSS increased followed by inoculation. Bull et al. (2005) also showed that no differences in market yield were detected between the inoculated and non-inoculated plants of strawberry.

Varying growing temperatures during the growing season commonly occur in many strawberry-production regions and inside the greenhouse without controlled environmental. During the harvest season temperature increased around 100 DAT (Figure 1) when it was observed reduction on fruit weight, size and quality related to sugar content and acidity (Table 2). High temperatures (24-32ºC) reduce strawberry flower formation (TAIMATSU et al., 1991; ODA & YANAGI, 1993; BRADFORD et al., 2010), fruit quality (KLAMKOWSKI & TREDER, 2008), fruit size and fruit weight (PALENCIA et al., 2013). Reproductive stage is very sensitive to adverse temperature conditions and pollen performance in strawberry is adversely affected by high temperatures (ZINN et al., 2010). Pollen viability has been shown to decline at temperatures above 25◦ C in certain short-day strawberry cultivars (LEECH et al., 2002; VOYIATZIS & PARASKEVOPOULOU-PAROUSSI, 2002; LEDESMA & SUGIYAMA, 2005).

Disconsidering the fruit from the first harvest, fruit size fit into the market level established by Souza (1972) of special type strawberry (8 g to 14 g) during harvest season. Our results demonstrate the potential of cv. Camarosa producing extra fruits according to classification, i.e. over 14 g (REBELO & BALARDIN, 1997), especially in October (115 and 126 DAT). In Brazil and Mercosul Technical Regulation of Strawberry Identity and Quality nº 85/96 use fruit diameter to classify strawberries (CANTILLANO, 2003; CEAGESP, 2006). According to this classification, strawberries Class 1 diameter must be bigger than 2.5 cm. During the harvest season fruit

could be classified as Class 1, except fruits from the first harvest (Table 2).

SST and TA are the most important indices of fruit quality, which are ubiquitously used, in standard quality controls (RESENDE et al, 2008; FAN et al., 2011). The flavour of strawberry fruits is determined by the balance of sugars and acids (KALLIO et al., 2000; KEUTGEN & PAWELZIK, 2007). The values of these variables determined in the present study are lower than the range of 8.5-14 considered appropriate balance of sweet-tart flavour notes in strawberry for human palatability (OREGON STRAWBERRY COMISSION, 2006). These values were only achieved at the last harvest when the temperature and PAR started to go higher. Fruit quality depends not only on physiological processes, but also the relationship between climate conditions (SHAW, 1990; KADER, 2002).

4.3 Total phenolic, anthocyanins and flavonols content and antioxidant activity

As demonstrated in this study the inoculation of AM fungi on strawberry plants, during transplant in soilless growth system, increases the content of secondary metabolites. However, different AM inoculum (native and isolates) vary in their efficiency to increase the synthesis of these compounds.

The accumulation of these metabolites is related to mycorrhization (ZENG et al., 2013). However, the mechanisms by AM fungi influence the production of these compounds are still not clarified (TOUSSAINT, 2007). Nutritional improvement of plants by

mycorrhizal symbionts plays an important role inducing secondary metabolites accumulation, as showed in strawberry and tomato fruits (CASTELLANO-MORALES et al., 2010; GIOVANNETTE et al., 2012). Increase in phenolic compounds content in mycorrhizal plants may be involve metabolic processes that can be mediated by a better absorption of P and N promoted by the symbiosis (TOUSSAINT et al., 2007; ZUBEK et al., 2010). Other authors suggest that this increase may be related to an increased activity of some enzymes (IBRAHIM & JAAFAR, 2011).

Phenolic compounds are secondary metabolites that constitute one of the most common and widespread groups of substances in plants (BRAVO, 1998). AM fungi inoculation can alter the production of phenolics within the host plants as reported in olive trees (GANZ et al., 2002), sweet basil (TOUSSAINT et al., 2007; LEE & SCAGEL, 2009), lettuce (BASLAM et al., 2013). Our results are in agreement with previous studies in strawberry (RIVERA-CHÁVEZ et al., 2012).

In the present study flavonoids and anthocyanins significant increase during harvest season (Figure 2 and 3) as well as the AM root colonization (Table 1). As shown *in vitro* studies plant flavonoids affect spore germination, growth of hyphae and stimulate root colonization (LAROSE et al., 2002). The same authors, pointed out the accumulation of flavonoids in plants were shown to be dependent on the development stage of the symbiosis as well as on species of AM fungi.

The increased of flavonoids in strawberry fruits followed by AM fungi inoculation was observed by other authors

(CASTELLANO-MORALES et al., 2010). Effect of AM fungi on this compound can be also observed in plant shoot of alfalfa (CATFORD et al., 2006), barrel medic (HARRISON & DIXON, 1993), red clover (KHAOSAND et al., 2008), soybean (MORANDI, 1996).

Anthocyanins in strawberry fruits are important both to assess the degree of maturity and are responsible for their colour, attributes that influence a great deal of the consumer's preference (WOJDYŁO et al., 2008). The effect of AM colonization on the concentration of anthocyanins was previously measured in strawberry fruits (CASTELLANOS-MORALES et al., 2010), who showed, for the first time, that symbiosis induces an increase in cyanidin 3-glucoside. The same response where observed later in strawberry (LINGUA et al., 2013), and also in cherry tomato (POULTON et al., 2001), basil (LEE & SCAGEL, 2009) and lettuce (BASLAM et al., 2011a,b).

Not only on phenolic compounds great differences were observed for inoculated and non-inoculated plants at last harvest, but also in the antioxidant activity, evaluated by FRAP (Figure 3). A significant increase in antioxidant activity of strawberry fruits (RAIOLA et al.; 2015) and artichoke (CECARRELLI et al., 2010) in the presence of AM fungi was also reported. Strawberries are good source of antioxidants and have higher antioxidant capacity compared to other fruits (SZETO et al., 2002). Anthocyanins together with flavonoids are in part responsible for the antioxidant capacity of strawberry fruits (AABY et al., 2007). This variable can be affected by agricultural practices, preharvest conditions, maturity, post-harvest handling and processing (KALT et al., 2001; WANG & ZHENG,

2001; WANG et al., 2002), cultivars and development stage (OLSSON et al., 2004).

Our results of the present study indicate that strawberry fruit quality can be improved by AM inoculation in soilless growth systems. The production of edible vegetables with high nutritional levels may lead to an increased nutrient ingestion by the population but not necessarily to an increased food intake (BASLAM et al., 2013). In addition, this technology, apparently, does not constitute any risk to human health (BASLAM et al., 2013; GIOVANNETTE et al., 2012). Increased quality, in terms of taste and nutritional value, can also become an additional target in agriculture, since, in recent years, consumers have sharpened their attention on all the aspects regarding the quality of foods and agricultural products in relation to health and environmental concerns.

5 CONCLUSION

The introduction of AM fungi into strawberry plants, during transplant in soilless growth system, increases the fruit quality parameters such as secondary metabolism compounds and also the antioxidant activity.

CHAPTER IV

ARBUSCULAR MYCORRHIZAL FUNGI IN STRAWBERRY PLANTLET PRODUCTION

ANA CLÁUDIA PEDERSEN

ABSTRACT - The use of plantlets of high physiological and sanitary quality is a critical step in strawberry crop production. However, most of the plantlets used are imported, what involves higher costs, limited supply, logistic problems of distribution, and the risk of plant pathogens introduction. The objective of this study was determined whether AM fungal pre-inoculation of strawberry micropropagated plants during the acclimatization stage benefit plant growth and clonal plant production in the field. Micropropagated strawberry plantlets (cv. Camarosa) were inoculated during the acclimatization stage and then transplanted to the field. After thirteen weeks plants were evaluated for establishment, growth and clonal propagation. Plants from all treatments showed AM fungal structures inside the roots after thirteen weeks in the field. However, AM fungal pre-inoculation did not result in growth benefits relative to non-inoculated controls. This absence of response was observed for both runner and clonal plant production. The present study was inconclusive regarding the benefit of pre-inoculation of micropropagated strawberry plants with arbuscular mycorrhiza fungi during acclimatization of plantlets on plant establishment, growth and clonal plant production in the field. However, the presence of AM fungi prior to field transplantation influences some root morphology parameters.

Key words: *Fragaria* x *ananassa* Duch., micropropagated plants, runners.

FUNGOS MICORRÍZICOS ARBUSCULARES NA PRODUÇÃO DE MUDAS DE MORANGUEIRO

RESUMO - O uso de mudas de alta qualidade fisiológica e sanitária é um passo crítico na produção do morangueiro. No entanto, a maioria das mudas utilizadas sáo importadas, o que envolve custos mais elevados, oferta limitada, problemas log[isticos e o risco da introdu;áo de fitopat[ogenos. O objetivo deste estudo foi determinar se a inoculação com fungos MA em mudas micropropagadas de morangueiro, durante a fase de aclimatização, traz benefícios na produção de mudas no campo. Mudas de morangueiro micropropagadas (cv. Camarosa) foram inoculados durante a fase de aclimatização e, posteriormente, transplantadas no campo. Após treze semanas as plantas foram avaliadas quanto ao estabelecimento, crescimento e produção de mudas. Plantas de todos os tratamentos apresentaram estruturas de fungos MA nas raízes após treze semanas no campo. No entanto, a pré-inoculação das plantas micropropagadas não resultou em benefícios no crescimento em relação as plantas controle. Essa ausência de resposta foi observada tanto na produção de de estolões e plantas clonais. O presente estudo foi inconclusivo quanto ao benefício da pré-inoculação de plantas de morango micropropagadas com fungos de micorrizas arbusculares, durante a aclimatação, no estabelecimento, crescimento e produção de mudas no

campo. No entanto, a presença desses microrganismos antes do transplante no campo influencia alguns parâmetros da morfologia das raízes.

Palavras-chave: *Fragaria* x *ananassa* Duch., plantas micropropagadas, estolão.

1 INTRODUCTION

Cultivated strawberry (*Fragaria* x *ananassa* Duch.) is a horticulture crop grown worldwide. Global strawberry production represents double the amount of all other berry crops combined (STEWART, 2011), which is explained due to high demand. As a result, strawberry is a crop of great economic and social importance in several countries and cultivation and production increase each year. From 2010 to 2013 world production increased 17.4% (FAOSTAT). This crop plays an important source of income for families of small and medium farmers in Brazil with approximately 0.5 to 1 ha (PAGOT & HOFFMANN, 2003) and production is around 100 thousand tonnes in an estimated area at 3,500 ha (CARVALHO, 2006; ANTUNES & REISSER JÚNIOR, 2007), mostly from soil cultivation (CARVALHO, 2006).

Plant propagation in horticulture systems usually starts from seedling, cuttings, grafting or tissue culture-derived plantlets. The use of plantlets of high physiological and sanitary quality is a critical step in the strawberry crop production. The asexual reproduction through stolons is used for commercially plantlet

production. For strawberry propagation, disease-free plantlets are first grown *in vitro* and, after a period of acclimatization, stock plants are grown in field soil nurseries for further production of plantlets (SANTOS & MEDEIROS, 2003).

Although these plants start out disease free, they are susceptible to pathogen attack. This technique allows to obtain a large number of healthy individuals with high productivity and genetically homogeneous in a short period of time (CARVALHO, 1999). However, the new plants (clonal plants) are rooted directly in the soil and it is at this stage that soil-borne pests and diseases can affect plantlet quality. To reduce or eliminate this problem strawberry plants have been grown in fumigated soils (i.e., with methyl bromide). Although fumigation controls weeds, nematodes, and soil-borne pathogens (WILHELM & PAULUS, 1980; YUEN et al., 1991) it also eliminates beneficial microorganisms, such as AM fungi. Due to its risk to the environment some countries banned the use of methyl bromide (BATCHELOR, 2002), as is the case of Brazil. Such ban represents a major challenge for strawberry crop that requires new alternatives. Reintroducing selected AM fungal species or communities during plant propagation may be important to minimize strawberry susceptibility to disease. The high incidence of diseases requires frequently application of fungicides. Thus, alternative solutions, such as the use of microorganisms, are necessary to reduce the use of chemical and ensure the safety and productivity of strawberry plantlets. The strawberry plants responses to inoculation with AM fungi have been studied for *Phytophthora fragaria*

(MURPHY et al., 2000b), *Rhizoctonia* solani (BAYÖZEN & YILDIZ, 2009) and *Fusarium oxysporum* (MATSUBARA et al., 2004).

Field inoculation of crop plants is impractical due to the technical difficulties and the large amount of inoculum required (WILLIANS et al., 1992). Vegetables and small fruit producers may have a more efficient option if using mycorrhizal technologies during plantlets production prior to outplanting. Inoculation of nursery plants may be an alternative for improving plant growth and quality (GIANINAZZI et al., 1989) and also can significantly influence the richness and diversity of the resident AM fungi communities (MUMMEY et al., 2009). *In vitro* propagated crops, such as strawberry, require much less AM inoculum than field crops and can be easily inoculated in the greenhouse, at the acclimatization stage of production, before transplanting into the filed for multiplication. After transplanting, the pre-colonized plants would establish a mycorrhizal hyphal network in the soil, which could serve as an inoculum source for the daughter plants (STEWART et al., 2005).

Proper use of AM fungi on plantlet production is beneficial (AZCÓN-AGUILAR & BAREA, 1997), as symbiosis is established before transplantation in the soil (ORTAS, 2008) what can bring more possibilities of obtaining benefits from AM inoculation, as shown for strawberry (CHÁVEZ & FERRERA-CERRATO, 1990). The AM inoculum can be mixed with the substrate, where plants coming from *in vitro* micropropagation, are rooted during acclimatization stage (NIEMI & VESTBERG, 1992a; WILLIANS et al., 1992; VESTBERG et al., 1994; RAI, 2001; STEWART et al., 2005).

Studies have shown that AM inoculation of micropropagated strawberry plants have benefited in terms of vegetative growth, where mycorrhizal plants produced more runners than the control plants under control conditions (HRSELOVA et al., 1990; CHÁVEZ & FERRERA-CERRATO, 1990; WILLIANS et al., 1992). But these responses can differ among the AM fungi species (NIEMI & VESTBERG, 1992; VESTBERG, 1992a; MURPHY et al., 2000a; ALARCÓN et al., 2001; TAYLOR & HARRIER, 2001) and strawberry cultivar (KHANIZADEH et al., 1995; WILLIANS et al., 1992; MARK & CASSELLS, 1996).

Not only is possible to obtain more runners with AM fungi but also earlier runners production (ALARCÓN et al. 2001). This way it is possible to avoid problems related to delay, or also could starts strawberry production earlier (UEMATSU, 1996) when the price in the market is higher (DURNER et al., 2002; DUVAL et al., 2004).

High fertilizer amounts are considered unfavourable for AM fungi formation (BIERMANN & LINDERMAN, 1983). It is possible to reduce fertilizer inputs during plantlets production by introducing beneficial microbial populations (SHARMA & ADHOLEYA, 2004) e.g. into the substrate during acclimatization stage. Strawberry has an extremely high P demand during reproductive stage, which could be met by AM fungi inoculation (DUNNE & FITTER, 1989).

The effects of mycorrhizal associations in agricultural systems are potentially beneficial, with a few negative reports on the growth of plants in the field. This may be related to the use of AMF species in commercial products, which are not present in cultivated

soil, leading to competition between indigenous and introduced species. Another aspect is the use of indigenous AM fungi for being more efficient as they are locally adapted to the soil conditions (SREENIVASA, 1992: OLIVEIRA et al., 2005b).

Many studies with micropropagated plants used as AM fungi inoculum contain *Glomus intraradices*. However, these studies do not show the effect of pre-inoculation of micropropagated strawberry plants on growth of the clonal plants produced in the field. We hypothesized that the presence of indigenous AM fungi community in micropropagated strawberry plantlets before transplanting in the field may benefits the establishment, growth and formation of clonal plants. The objective of this study was determined whether AM fungi inoculation on strawberry micropropagated plants during acclimatization stage benefit plant establishment, growth and clonal plants production in the field.

2 MATERIAL AND METHODS

2.1 Plant material and mycorrhizal inoculum

Micropropagated strawberry plantlets (cv. Camarosa) propagated by tissue culture, were obtained from NLARS, University of Guelph.

Plantlets were inoculated with AM fungi during acclimatization stage. Inoculum treatments consisted of an indigenous AM fungi community from a strawberry farm (Sault Ste. Marie, ON, Canada, 46º32’53.5”N, 84º27’46.6”W) and a commercially available inoculant Myke® Pro WP. The indigenous inoculum consisted of soil

including pieces of strawberry roots. The commercial inoculant contained a single isolate of *Glomus intraradices* (800 spores per g; produced by Premier Tech Biotechnologies, Rivi-ère-du-Loup, Quebec, Canada, for the purpose of being used in agricultural systems) was used for this treatment.

For indigenous AM fungi community treatment soil inoculum was mixed with the substrate (1:2, v:v). Commercial product treatment and control also received the same portion of autoclaved soil inoculum (two cycles, 121 ºC for 1h, 24h interval).

The commercial product was diluted in distilled water, and 1.5 mL of this solution was applied to each pot, representing 1.2 g of the product. This solution was sterilized (100 ºC for 15 min) and applied in both indigenous AM fungi community and control treatments.

To correct for differences in non-AM microbial communities, each experimental unit received a 20 mL filtered washing comprised of extract from soil used for indigenous AM fungi community inoculum. To prepare this wash, 1 kg of soil was diluted in 2 L water and mixed. The mix was allowed o settles for 20 min and decanted through 20 µm sieve to retain AM fungi infectious propagules while bacteria and other non-AM fungi spores pass through.

2.2 Experimental design

Treatments consisted of: indigenous AM fungi community; commercial product and non-mycorrhizal control. The

treatments were distributed in a completely random design, with twenty repetitions.

After acclimatization phase from a total of twenty replicates, only 6, 13 and 14 plants from indigenous AM fungi community, commercial product and control treatments survived, respectively. This way, there were not enough plants for evaluation before transplanting in the field.

After thirteen weeks in the field five plants from indigenous AM fungi community, ten plants from commercial product and thirteen plants from control treatment survived.

2.3 Experiment setup

The experiment was divided in two phases: 1) micropropagated strawberry plants were pre-inoculated with AM fungi during acclimatization stage in a growth chamber, for one month; 2) after, these plants were transplanted into the field (46º32'54.06"N 84º27'45.39"W, elevation 235 m) for growth of the clonal plants.

Healthy regenerated plantlets with well-developed roots were removed from the culture medium. The roots were carefully washed under distilled water to remove adhering culture media. These plantlets were transferred to 400 mL containers (Stuewe and Sons Inc, Tangent, Oregon) filled with sterilized substrate (two cycles, 121 ºC for 1h, 24h interval) containing turface, sand and field soil (1:1:2, v:v:v) for pre-inoculation and acclimatization. All plants were covered with a transparent plastic cup to provide a high relative humidity until the plant established its roots and appeared healthy (two weeks). The

plants were kept in the growth chamber under controlled conditions (20-23 ºC; 55% relative humidity) and watered every day. The cover was gradually removed after this period. The plantlets did not receive any fertilizer during acclimatization.

The plantlets were transplanted into the field one month after acclimatization. The field site was the same strawberry farm from where we collected the soil (46º32'53.5"N, 84º27'46.6"W, 192 m elevation). Each experimental unit had 1 m^2 area.

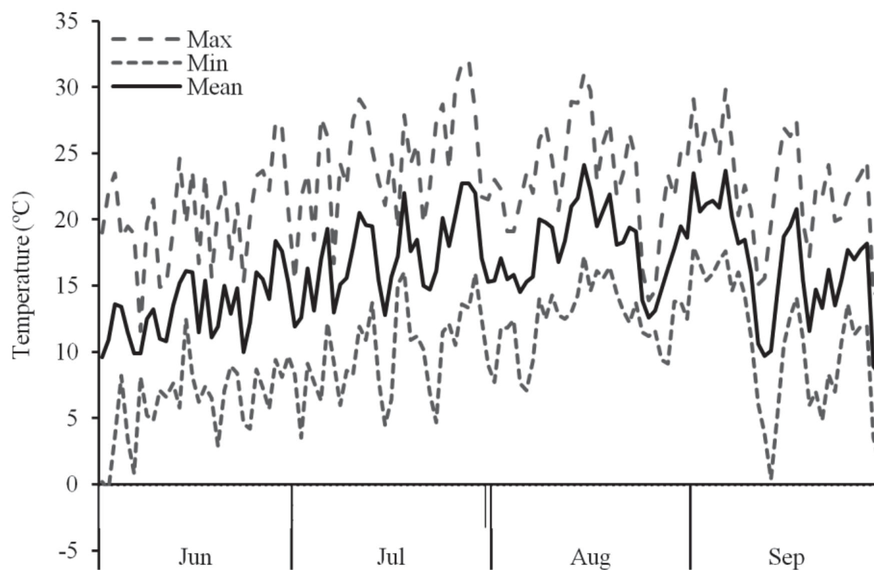

Figure 1. Temperature during the experiment. Sault Ste. Marie, 2015. Source: climate.weather.gc.ca/

2.5 Harvest and response variables

After thirteen weeks of growth in the field, plants and clonal plants were harvested.

After harvesting, crown diameter (mm) was measured using a digital caliper. Leaf number was recorded. Plants were then

divided into roots, crowns and leaves. Fresh biomass was measured using a digital scale. For dry biomass evaluations, shoot, roots and crowns were placed in paper bags and dried in an oven at 65 °C to constant weight.

Before drying the roots, morphological parameters were determined using WinRhizo Pro image analysis software (Regent Instruments Inc., Quebec City, QC). Root length, volume, average diameter (very fine roots, Ø < 0.5 mm; fine roots, 0.5 < Ø > 1.0 mm; fibrous roots, 1.0 < Ø > 1.5 mm; coarse roots, Ø > 1.5 mm), surface area and number of tips and forks were automatically analyzed using this software. Specific root length (SRL) was calculated from the length divided by root biomass, and root tissue density (RTD) from root dry biomass divided by estimated volume.

AM root colonization was determined in the roots after thirteen weeks of growth in the field. Root samples were washed thoroughly with running tap water to remove the adhered soil particles and cut into small pieces of about 1 cm following the method by Phillips & Hayman (1970). AM root colonization was quantified placing 1 cm segments onto a microscope slide and observed under microscope (TROUVELOT et al., 1986). Accordingly, 100 intersections were quantified for each sample and the presence of any vesicles, arbuscules, or internal hyphae was assessed and percent root colonization was calculated from the number of colonized intersections out of total root intersections.

2.6 Statistical analysis

Statistical analyses were performed using COSTAT (CoHort Software, 2003). The effects of inoculation were evaluated using one-way analysis of variance (ANOVA). Mean comparisons were made using Tukey-Kramer test, differences at $p \leq 0.05$ were considered significant.

3 RESULTS

Plants from all treatments had AM fungal structures inside the roots after thirteen weeks in the field. Plants inoculated with indigenous community showed higher level of AM colonization compared to control plants. However, no clear trend was found among the treatments (Figure 2).

Pre-inoculation failed in producing any positive effect on plant biomass in the field (Table 1). In addition, the same response was observed for runner and clonal plant production and growth parameters (Table 2).

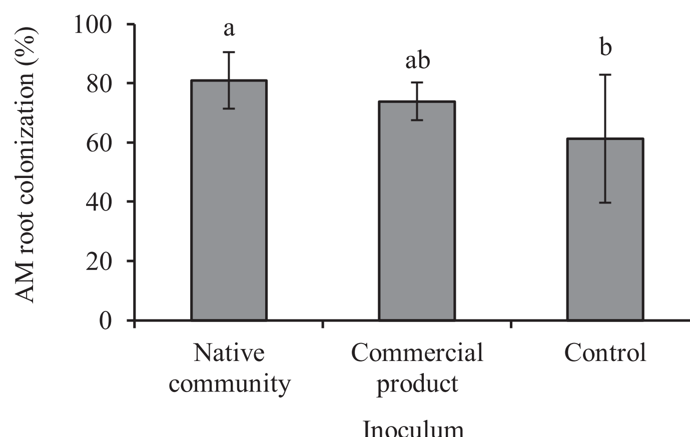

Figure 2. Arbuscular mycorrhizal (AM) colonization of strawberry plants inoculated with arbuscular mycorrhiza fungi during acclimatization, after 13 weeks in the field. Data (means ± SD) followed by the same letter above the bars are not significantly different at Tukey-Kramer test (P≤0.05). Algoma University, Sault Ste. Marie, 2015

Table 1. Leaf number, crown diameter, fresh and dry biomass of leaves, crown and roots of strawberry plants (cv. Camarosa) inoculated with arbuscular mycorrhiza fungi during acclimatization, after 13 weeks in the field. Algoma University, Sault Ste. Marie, 2015

Attribute		Treatment		
		Indigenous community	Commercial product	Control
Leaf number plant^{-1}		6.00 ± 0.70^{ns}	8.40 ± 2.46	7.69 ± 2.62
Crown diameter (mm)		7.84 ± 1.97^{ns}	9.55 ± 2.01	9.37 ± 1.83
Fresh biomass (g plant^{-1})	Leaves	8.00 ± 3.46^{ns}	14.38 ± 5.83	15.13 ± 6.79
	Crown	0.54 ± 0.21^{ns}	0.86 ± 0.38	0.93 ± 0.45
	Roots	4.08 ± 2.58^{ns}	6.50 ± 3.42	5.99 ± 2.62
Dry biomass (g plant $^{-1}$)	Leaves	2.25 ± 1.63^{ns}	3.52 ± 1.24	3.36 ± 1.59
	Crown	0.09 ± 0.04^{ns}	0.16 ± 0.8	0.17 ± 0.09
	Roots	0.56 ± 0.40^{ns}	0.71 ± 0.46	0.62 ± 0.30

Values are means ± SD. ns: non-significative according to Tukey-Kramer test (P≤0.05).

Table 2. Runner and daughter plants number, fresh and dry biomass of strawberry plants (cv. Camarosa) inoculated with arbuscular mycorrhiza fungi during acclimatization, after 13 weeks in the field. Algoma University, Sault Ste. Marie, 2015

Attribute		Treatment		
		Indigenous community	Commercial product	Control
Number plant $^{-1}$	Runners	1.80 ± 2.16^{ns}	3.50 ± 1.84	2.54 ± 2.14
	Clonal plants	1.80 ± 2.49^{ns}	4.90 ± 4.25	3.15 ± 3.34
Fresh biomass (g plant^{-1})	Runners	1.58 ± 1.81^{ns}	3.73 ± 2.36	3.70 ± 3.86
	Clonal plants	2.84 ± 4.35^{ns}	10.15 ± 12.28	7.09 ± 9.50
Dry biomass (g Plant^{-1})	Runners	0.43 ± 0.61^{ns}	0.81 ± 0.53	0.70 ± 0.71
	Clonal plants	1.20 ± 2.22^{ns}	2.81 ± 3.65	1.85 ± 2.63

Values are means ± SD. ns: non-significative according to Tukey-Kramer test ($P \leq 0.05$).

Plants inoculated with indigenous community had higher RTD (Table 3) and greater proportions of root length of very fine roots (Ø < 0.5 mm) and the proportion of coarse roots (Ø > 1.5 mm) decreased (Figure 3).

There was no relationship between AM root colonization and growth parameters as well as root morphology (Table 4).

Table 3. Root morphology attribute of strawberry plants (cv. Camarosa) inoculated with arbuscular mycorrhiza fungi during acclimatization, after 13 weeks in the field. Algoma University, Sault Ste. Marie, 2015

Root attribute	Treatments		
	Indigenous community	Commercial product	Control
Length (cm)	1211.85 ± 465.24^{ns}	1232.65 ± 369.60	1178.69 ± 330.70
Project area (cm^2)	67.20 ± 47.93^{ns}	107.39 ± 54.84	91.14 ± 41.89
Surface area (cm^2)	211.12 ± 150.60^{ns}	337.38 ± 172.29	286.33 ± 131.61
Diameter (mm)	0.51 ± 0.21^{ns}	0.89 ± 0.50	0.73 ± 0.22
Root volume (cm^3)	3.31 ± 3.84^{ns}	8.99 ± 8.39	5.86 ± 3.75
Tips	7990 ± 4385^{ns}	6751 ± 3989	5472 ± 2593
Forks	25324 ± 15728^{ns}	19210 ± 8937	18242 ± 8448
SRL (cm g^{-1})	330.03 ± 78.93^{ns}	242.04 ± 130.69	243.95 ± 144.26
RTD (g cm^{-3})	0.26 ± 0.23 a	0.12 ± 0.06 b	0.14 ± 0.09 ab

Values are means ± SD. SRL: specific root length; RTD: root tissue density. Identical letters in the same row indicate no significant differences between treatments ($P \leq 0.05$). ns: non-significative.

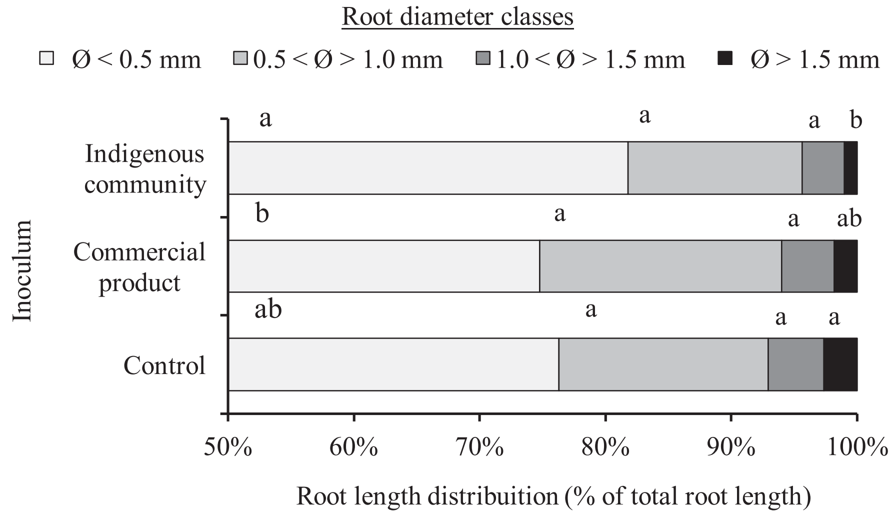

Figure 3. Root length distribution on the root diameter classes of strawberry plants (cv. Camarosa) inoculated with arbuscular mycorrhiza fungi during acclimatization, after 13 weeks in the field. Different letters within each AM inoculum and root-diameter class indicate significant differences according to Tukey-Kramer test (P≤0.05).

Table 4. Correlation between arbuscular mycorrhizal root colonization and growth attribute and root morphology. Algoma University, Sault Ste. Marie, 2015

Attribute	Coefficient of correlation (%)	P
Growth variables		
Fresh biomass (g)		
Leaf	0.03^{ns}	0.8789
Crown	-0.00^{ns}	0.9966
Root	0.00^{ns}	0.9838
Runner	0.18^{ns}	0.3396
Clonal plants	0.15^{ns}	0.4437
Dry biomass (g)		
Leaf	0.13^{ns}	0.5162
Crown	0.03^{ns}	0.8978
Root	0.06^{ns}	0.7704
Runner	0.22^{ns}	0.2564
Clonal plants	0.18^{ns}	0.3567
Leaves plant^{-1}	-0.15^{ns}	0.4189
Crown diameter	-0.02^{ns}	0.9096
Runners plant^{-1}	0.23^{ns}	0.2306
Clonal plants plant^{-1}	0.19^{ns}	0.3221
Root morphology		
Length (cm)	0.15^{ns}	0.4407
Surface area (cm^2)	0.09^{ns}	0.6660
Project area (cm^2)	0.09^{ns}	0.6659
Average diameter (mm)	0.01^{ns}	0.9448
Root volume (cm^3)	0.05^{ns}	0.8038
Tips	0.24^{ns}	0.2181
Forks	0.19^{ns}	0.3269
RTD (g cm^{-3})	-0.21^{ns}	0.2916
SRL (cm g^{-1})	0.18^{ns}	0.3587

SRL: specific root length; RTD: root tissue density. ns: non-significative ($p \leq 0.05$).

4 DISCUSSION

It was hypothesized that pre-inoculation with AM fungi in micropropagated strawberry could influence positively establishment, growth and clonal production in the field. Plant establishment and clonal production were unaffected by inoculation and only some root growth parameters were promoted by AM fungi.

All plants were colonized by AM fungi after thirteen weeks of growing in the field. Control plants showed lower values of AM root colonization, which was expected considering that these plants only established the symbiosis with AM fungi after being transplanted into the field. The lowest value of AM root colonization in control plants indicates that pre-inoculation was efficient and could offer potentially benefits (ORTAS, 2008).

In some cases, high rates of AM root colonization result in no benefit in terms of biomass to the host plant (LOVATO et al., 1994). The lack of benefits can be due to the fungus representing an additional drain of photoassimilates (LOCATELLI & LOVATO, 2002), which may have a negative impact on growth (HRSELOVÁ et al., 1990).

Contrary to the results, the use of AM fungi at acclimatization of plantlets has shown positive growth results. Micropropagated plantlets of apple, plum and peach resulted in additional new leaves in the presence of mycorrhiza compared to control plants (SBRANA et al., 1994). Other reports show beneficial effects on growth (VESTBERG, 1992b; BORKOWSKA, 2002) and mycorrhizal plants producing more runners than the control plants (CHÁVEZ & FERRERA-CERRATO, 1990; BOTHAM et al., 2009).

These benefits may be related to nutritional and hormonal changes that the symbiosis promotes (VARMA & SCHUEPP, 1994). One factor that may have contributed to the absence of responses to growth and runner production may have been low temperatures during the experiment period.

The mycorrhizal association did not improve the survival rate of micropropagated plants after acclimatization and in the field, which is in contrast to the findings by Varma & Schuepp (1995) also on strawberry. After acclimatization only 30, 65 and 70% of plants from indigenous AM fungi community, commercial product and control treatments survived, respectively.

The micropropagation technique stands out from conventional methods. However, the exchange of plants from *in vitro* to *ex vitro* conditions (acclimatization) can result in low survival rate (CALVETE e al., 2000). Among the possible causes of low survival rate in acclimatized *ex vitro* plants is water stress caused by the change of environment (BRAINERD & FUCHIGAMI, 1981) and limited photosynthetic capacity (PREECE & SUTTER, 1991). Another benefit of AM fungal inoculation during the acclimatization period is reducing the osmotic potential of the plant (VARMA & SCHUEPP, 1994) and increasing plant tolerance to drought stress (BORKOWSKA, 2002).

Overall, plants inoculated with AM fungi presented more tips and forks per plant (Table 3). This is consistent with data indicating that when root tips are colonized by the mycorrhizal fungi, the roots can stop their linear growth while secondary (diameter) growth continues (BERTA et al., 1993; SHISHKOVA et al., 2008).

There are several studies on the influence of AM fungi inoculation on root morphology (BERTA et al., 1993; ATKINSON et al., 1994; FAN et al., 2011). We found that the percentage of thinner roots (Ø < 0.5 mm) tended to increase whereas coarse roots decreased through mycorrhizal inoculation with indigenous community (Figure 3). The same treatment showed the highest value of AM root colonization (Figure 2). Contrary to the results, AM fungi form associations within cortical cells along the root axis, such that thicker roots with a thick cortex may be able to support more AM per unit of root length or mass (BRUNDRETT, 2002; GUO et al., 2008).

Contrary to the results, Fan et al. (2011) pointed out that the inoculation of AM fungi significantly increased root length of medium (0.5 < Ø > 1.5 mm) and coarse (Ø > 1.5 mm) roots on strawberry plants. The morphology of root systems is altered by AM fungi in a structural, spatial, quantitative, and topological manner (SMITH & READ, 2008; FAN et al., 2011).

Thinner roots represented more than 80% of the total root length in plants inoculated with indigenous AM fungi (Figure 3). Long fine roots (Ø < 2 mm) are functionally responsible for the spread and stability of the fine root system and for the nutrient transport to the coarse roots. Fine roots (Ø ≤ 2 mm) are the major part of the root system involved in absorbing water and nutrients (SHI et al., 2007).

The effect of indigenous AM fungi community pre-inoculation on root length and fine roots, and, consequently, on high SRL, is believed to be the below-ground equivalent of thinner leaves (i.e., smaller leaf mass area), which require less energy to produce (WITHINGTON et al., 2006). Specific root length is probably the

most commonly measured morphological parameter of roots because it is highly responsive to changes in carbon balance (OSTONEN et al., 2007). These authors pointed out that increasing SRL is an intensive strategy, which increases the volume of soil exploited per unit biomass invested in the fine roots. Specific root length is a complex parameter that can be influenced both by root diameter and tissue density (COMAS & EISSENSTAT, 2004). Indeed, correlation analyses reveal a significant relationship with root diameter ($r = -0.72$, $p<0.001$) and RTD ($r = 0.55$, $p=0.003$). Thinner roots with higher SRL may generally be less dependent on mycorrhiza if their morphology is better suited for nutrient acquisition without mycorrhiza (BRUNDRETT, 2002), although exceptions could be found as in our study and by Siqueira & Saggin Junior (2001)

There was a significant difference in the RTD, but insignificant difference in average diameter among the treatments (Table 3). The root diameter decreases and RTD increases at the same time is evidenced by the negative correlation between the variables ($r = -0.79$, $p<0.001$).

Since, the benefits of inoculation with AM fungi are varied and dependent, more studies should be carried out with different combinations of AM species, strawberry cultivars and study site to better determine the extent to which pre-inoculation with these microorganisms is important. Because our results show some evidence for AM inoculation effects on root morphology, we propose that special attention should be placed in understanding how these changes may influence strawberry production.

5 CONCLUSIONS

The present study was inconclusive regarding the benefit of pre-inoculation of micropropagated strawberry plants with arbuscular mycorrhizal fungi during acclimatization of plantlets on plant establishment, growth and clonal plant production in the field. However, the presence of AM fungi prior to field transplantation influences some root morphology parameters.

CONCLUSION

This study shows the occurrence of mycorrhizal fungi in soils under strawberry crop and the most frequently species are *Claroideoglomus etunicatum* and *Funneliformis mosseae*. Also shows that the introduction of mycorrhizal fungi in soilless crop system is a tool which influences the leaf appearance rate and benefits fruit quality. In addition, inoculation of plantlets in this system may turn strawberry production more sustainable.

FINAL CONSIDERATIONS

This study reports for the first time the occurrence of arbuscular mycorrhizal fungi species associated with strawberry (Chapter I). This survey showed differences in the occurrence and effectiveness of this fungus in soils under strawberry crop. These data contribute to the knowledge of fungi occurrence in Rio Grande do Sul. However, the samples are from a restricted region (Ibirubá, Tapera and Passo Fundo) with strawberry crop. Thus, more research is needed in a larger sample area as well as in different culture systems.

This research has practical implications for strawberry crop as the identification of fungal species will contribute to the production of commercial inoculants and/or developed by the farmers in their property (on farm inoculum). On the other hand, it deduced the possibility of introducing the inoculum both at the time of transplantation as in the plantlets acclimatization phase (Chapter IV), to better establish of the symbiosis.

Overall, these results showed that inoculation with mycorrhizal fungi mycorrhizal improved nutritional quality of strawberry fruits (Chapter III), but without answers on growth and yield (Chapter II). These results suggest the need to find an inoculum that can aggregate yield and quality in the strawberry crop or conduct crop for another year. Since producers are shifting production in the soil to soilless crop system, due to reduction of labor and the practicality of this system. Also it is suggested that studies using these fungi under conditions of reduced fertilizer without loss to the quality and yield of the fruits.

Pre-inoculation with AM fungi in micropropagated plantlets showed differences in mycorrhizal colonization with effect on the root diameter (Chapter IV). However, there was no positive influence on the establishment of the plantlets in the field, as well as in the production of clonal plants. To clarify the reason for the absence of positive results the same experiment was repeated at University of Passo Fundo.

Nevertheless, much remains to be elucidated about the potential use of AM fungi in soilless crop systems and how we can use these microorganisms in order to maximize yield and quality of fruits and at the same time minimize environmental impacts. The benefits promoted by symbiosis on the growth and health of plants point to the use of this practice in horticulture crops as a sustainable tool, which is desired in any crop system, especially in strawberry, which requires high inputs.

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