Agriculture and Natural Resources 52 (2018) 388–392

Contents lists available at ScienceDirect

Agriculture and Natural Resources

journal homepage: http://www.journals.elsevier.com/agriculture-and-natural-resources/

Short Communication

Effect of agrochemicals on endophytic fungi community associated with crops of organic and conventional soybean (*Glycine max* L. Merril)

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ARTICLE INFO

Article history:
Received 27 July 2017
Accepted 10 May 2018
Available online 18 October 2018

Keywords:
Endophyte
Environment
Fenoxaprop-P-Ethyl
Triflumuron
Pesticide

ABSTRACT

The use of agrochemicals in crops worldwide can have important impacts on the environment where they are used, including the rhizosphere. The effects were assessed of triflumuron and fenoxaprop-P-ethyl on the fungal endophyte community associated with soybean in conventional and organic crops, at 55 d and 75 d after seed germination. Diversity, richness, and evenness decreased in the conventional crop. The number of genera of fungi isolated also decreased from 29 in organic soybean to 20 in conventional soybean. The occurrence of genera was evenly distributed in organic soybean. In conventional soybean, there was an increased frequency of some genera over others, revealing an ecological imbalance induced by the use of the tested pesticides.

Introduction

Soybean is one of the most important commodities worldwide; it is the main oilseed produced annually in the world, with about 348 million t in the 2016/17 harvest (DEAGRO/FIESP, 2017). Its wide use is due mainly to its high nutritional value and the health benefits it offers (Zhang et al., 2011; Cho et al., 2013). Two main cropping systems are used in this culture: organic and conventional systems. Organic systems are defined as a sustainable practice characterized by maintaining and protecting natural resources by abolishing the use of agrochemicals, while conventional farming systems are characterized by management practices that include pesticides, which often can be harmful to consumers and to the environment (Bettiol et al., 2002). Even though the pesticides are generally effective against their targets, they usually are not specific to them, and therefore can damage the rhizosphere (Nettles et al., 2016) and cause economic losses that may go beyond environmental contamination, such as medical expenses due to poisoning (Oliveira et al., 2014). The use of agrochemicals in conventional systems can cause contamination of soil and water (Laabs et al., 2000), the appearance of pesticide-resistant pests (Popp et al., 2013) and changes in the community of fungal endophytes associated with crop plants (Tkaczuk and Mietkiewski, 2005; Stuart, 2006).

Fungal endophytes colonize healthy plant tissues during at least part of their life cycle, without producing disease symptoms on the plant (Petrini et al., 1992; Azevedo and Araújo, 2007), and often develop a symbiotic relationship with the host (Alvin et al., 2014). Some endophytes are well known for their biotechnological potential, for example, for the production of certain drugs such as the chemotherapic taxol (Strobel, 2003) and new antibiotics (Strobel et al., 2004). Some endophytes can also increase the plant's defense against pests and phytopathogens (Abrahão et al., 2013), as they increase the production of insecticidal compounds and promote plant growth (Strobel and Daisy, 2003).

The endophytic community can be affected naturally by changes in environmental conditions and soil types. They can also be affected by the application of agrochemicals (Stuart et al., 2010) which can affect the plant life cycle and metabolism. Thus, the objective of the current study was to assess the effect of the application of agrochemicals on the fungal endophyte community associated with soybean.

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https://doi.org/10.1016/j.anres.2018.10.005

Materials and methods

Plant sowing and treatments

Soybean (*Glycine max* (L.) Merril) of the BR36 variety was planted by Tozan Alimentos Orgânicos S/A, in the municipality of Piraí do Sul, Parana state, Brazil (24°31′34″S 49°56′55″W). The experiment was conducted in two separated fields of 3 ha each, with one using an organic culture system and one using conventional practices. Both fields were treated with the "Supermagro" biofertilizer [Silva and Carvalho, 2000; MAPA (Ministry of Agriculture, Livestock and Food Supply), 2016] and baculovirus biocontrol 40 d after germination. The conventional production crop was also treated with the systemic herbicide Podium S (Bayer; active ingredient: fenoxaprop-P-ethyl) of the aryloxyphenoxy propionic acid group at 50 d of growth, and the insecticide Certero (Bayer; active ingredient: triflumuron), a chitin synthesis inhibitor of the benzoylurea group, at 70 d of growth. Both were applied following the manufacturer's recommendations. These agrochemicals were chosen due to their wide use in soybean cultivation. Leaf samples were made at the V4 vegetative stage (4 knots on the main stem beginning with the interfoliate node at approximately 55 d after seed germination) and at the reproductive stage R4 (flower at the penultimate node of the apex region with a completely unrolled leaf at approximately 75 d after the germination of the seeds).

Field history and conditions

Culture rotation between maize (*Zea mayz* L.) and soybean (*Glycine max* L.) was used to guarantee soil recovery, maintenance, improvement of natural resources, and aiming at raising productivity. The crop soil (Cambisol Haplic Typical Tb Dystrophic; CXbd), had good organic matter content, favorable relief and can be used indiscriminately with annual or perennial crops, as long as properly corrected and fertilized.

Supermagro recipe

According to Silva and Carvalho (2000) and MAPA (2016), 200 L of Supermagro biofertilizer includes: 2 kg of zinc sulfate, 2 kg of calcium chloride, 2 kg magnesium sulfate, 300 g of manganese sulfate, 50 g of cobalt sulfate, 100 g of sodium molybdate, 1 kg of boric acid, 1,5 kg hydrated lime, 8 L of milk, 8 L of cane molasses or 4 kg of brown sugar, 200 g of bone flour, 50 kg de fresh manure and water to make the mix up to 200 L. It may be added to plants, vegetables, medicinal plants and fruits as fertilizer. Before its use, the biofertilizer must be fermented and bioestabilized (cured). It is important to use gloves during the handling of the Supermagro biofertilizer. The magnesium sulfate used for fertilization and soil correction must be from a natural source.

Isolation of endophytic fungi

One hundred plants from each treatment were sampled 15 d and 35 d after the application of the pesticides (corresponding to 55 d and 75 d after germination). Healthy leaf surfaces (10 leaves per plant) were disinfected with a series of baths in 70% ethanol (1 min), 2% sodium hypochlorite (3 min), 70% ethanol (30 s) and finally three baths in sterilized distilled water. The leaves were fragmented into 5–8 mm^2 pieces and transferred in an aseptic environment to Petri dishes, six pieces per dish, containing potato, dextrose agar (PDA) culture medium (Himedia; Mumbai, India) supplemented with penicillin and tetracycline (100 μg/mL) to prevent bacterial growth. To provide evidence of the effectiveness of disinfection, 100 μL of the distilled water used for the last bath was also plated in Petri dishes with PDA medium.

The dishes were incubated at 28°±1 °C with a 12 h photoperiod and examined at regular intervals for up to 30 d for the formation and growth of colonies. The emerging colonies were marked and immediately transferred to a new plate, then purified and grouped according to their morphological characteristics. The isolated fungi were identified using microculture, based on the reproductive structures of the anamorphous (Kern and Blevins, 1999). Statistical analysis was based on Tukey's multiple comparison tests and analysis of variance (Camatti-Sartori et al., 2005).

Diversity, richness, and evenness

Taxon diversity, richness, and evenness were quantified using Shannon's (H'), Margalef's € and Sheldon's €, indices respectively. The maximum theoretical diversity (H' *max*) was determined according to Krebs (1999). The similarity among the endophytic communities was also determined using Horn's index (Horn, 1966). This parameter was based on the composition of the community and species abundance, producing community clusters using the unweighted pair-group method with arithmetic averages.

Results and discussion

Isolation of endophytic fungi

There was a significant difference in the number of isolate fungi in the first and second collecting events, both for organic and conventional crops. In the organic crop, 1760 endophytic fungi isolates were obtained after 55 d post-germination and 654 isolates after 75 days post-germination. For the conventional soybeans, 1783 fungi were isolated after 55 d post-germination and 505 after 75 d post-germination. There was no statistical difference between the two farming practices in this aspect. However, it was observed that the isolation frequency was significantly different considering the time of sampling (55 d and 75 d post-germination). Observed differences could be attributed to the leaf or plant age, which may influence not only the number of fungi isolated but also the genus-level diversity (Giauque and Hawkes, 2016). Pimentel et al. (2006) also observed a decrease in the number of endophytes between the first and second sampling events in a study on soybeans planted under field and greenhouse conditions and they attributed those changes to plant age.

Since there was no significant difference in isolation frequencies between the farming systems, both can be considered favorable to colonization by endophytic fungi. However, there were important differences in the genera isolated from the organic and conventional soybean crops. In total, 37 morphotypes of fungal endophytes were isolated from organic soybeans, with 29 distinct genera. Eight morphotypes were indicated as *Micelia sterilia* because they did not produce spores in the culture medium. For conventional soybeans, 25 morphotypes were identified, being 20 distinct genera and five *Micelia sterilia* (Table 1). These differences could be associated with the effects of the agrochemicals on the overall environment, leading to changes in the endophytic microbiota and favoring the prevalence of some genera over others. Campisano et al. (2014) recorded changes in the composition of endophytic bacteria communities in grape when comparing organic and integrated management systems which included agrochemicals. Similar changes in microbiota have also been described for corn and soybean seeds (Nettles et al., 2016), where the use of pesticides significantly affected the abundance of genera and induced changes in the species composition of endophytes in these cultures.

Table 1
Isolation frequency of endophytic fungi isolated from organic and conventional soybean leaves, 55 d and 75 d after seed germination.

Genus	Organic		Conventional	
	55 d (%)	75 d (%)	55 d (%)	75 d (%)
Acremonium sp.	3.6	0.9	6.8	5.1
Alternaria sp.	11.4	19.7	76.4	64.6
Aspergillus niger	0.3	0.8	0.3	0.4
Aspergillus sp.	–	–	–	0.8
Aureobasidium sp.	1.3	–	–	–
Bipolaris sp.	7.6	10.9	1.7	2.4
Bleptosporium sp.	–	0.9	–	–
Chaetomium sp.	–	–	–	1.4
Cladosporium sp.	2.3	2.0	–	–
Colletotrichum sp.	2.8	–	–	0.8
Drechslera sp.	2.4	3.1	0.6	3.8
Epicoccum sp.	0.6	–	1.7	–
Fusarium sp.	10.9	5.4	1.0	0.4
Geotrichum sp.	0.6	6.4	0.6	–
Harpographium sp.	–	1.5	–	–
Idriella sp.	1.1	–	0.1	–
Micelia sterilia I	1.0	0.9	–	–
Micelia sterilia II	1.0	–	0.3	–
Micelia sterilia III	1.4	0.2	–	–
Micelia sterilia IV	0.9	0.6	0.6	–
Micelia sterilia V	1.4	–	0.4	–
Micelia sterilia VI	–	4.6	–	0.2
Micelia sterilia VII	–	7.8	–	2.0
Micelia sterilia VIII	–	2.9	–	–
Nigrospora sp.	7.2	3.8	2.9	11.3
Paecilomyces sp.	–	2.3	–	–
Penicillium sp.	4.4	3.8	–	0.2
Pestalotiopsis sp.	4.5	–	6.3	–
Phoma sp.	2.3	–	–	0.6
Pyricularia sp.	2.5	5.8	–	–
Rhizoctonia sp. (binucleada)	13.5	7.2	–	–
Rhizoctonia sp. (multinucleada)	5.7	4.3	–	2.0
Sclerotium sp.	1.1	–	–	–
Scopulariopsis sp.	–	1.4	–	–
Scytalidium sp.	4.0	–	–	–
Stemphylium sp.	–	0.9	–	–
Trichoderma sp.	–	1.1	–	2.0
Trichophyton sp.	1.8	–	–	0.2
Trichothecium sp.	2.4	0.9	–	–
Verticillium sp.	–	–	0.2	–
Total (%)	100	100	100	100
Total number of isolates	1760	654	1783	505
Total number of morphotypes	28	26	15	17

The effects of herbicides and biocides over non-target communities have been studied for some time. Wyss et al. (2004) reported that the imidazolinone group of herbicides induced changes in colony coloration and in the germination of the endophyte *Phomopsis amaranthicola*. The insecticides amitraz, hexythiazox and phosalone, and the herbicide pendimethalin, inhibited the *in vitro* growth of the entomopathogenic fungus *Hirsutella* sp. (Tkaczuk and Mietkiewski, 2005). Such works illustrate the negative effect of pesticides on non-target communities such as fungal endophytes.

Endophytic microbiota is directly correlated with plant growth and health. Xia et al. (2015), showed that endophytic bacteria, isolated from tomato fruits, were able to promote plant growth and biomass accumulation with agroecosystem application. Another study showed that endophytic fungi improved plant tolerance and reduce cadmium uptake, which may be recommended for phytostabilization in crop fields (Khan et al., 2017). González-Teuber (2016) demonstrated that endophytic fungi promoted plant protection against natural enemies. On the other hand, Worthington (2001), found significant nutritional differences in organic fruits, vegetables, and grains, compared to conventional culture, with the organic crops containing more minerals and vitamins, as iron, magnesium, phosphorus and vitamin C, and less heavy metals than conventional crops. Similar findings were made by Brandt and Molgaard (2001) and Lima and Vianello (2011), which evidenced a higher amount of nutrients in organic than in conventional crops, besides their benefit to human health. Considering these studies, it seems that highly diverse endophytic microbiota is closely related to human and plant health and higher nutritional value, plant protection and plant growth, once a diverse endophytic microbiota is related to an organic crop. In soybean, further studies must be carried out to evaluate the effect of pesticides application on plant health, nutritional value, and growth.

The identification of endophytic isolates indicated two distinct communities. The genus *Alternaria* was the most common in both organic and conventional soybeans (Table 1), representing 76.4% of the endophyte isolates from conventional plants right after the application of the pesticides (first sampling event). On the other hand, this genus represented only 11.4% of the isolates from organic plants in the same sampling period. This prevalence may have been due to the imbalance caused by the agrochemical application on conventional soybean, where the growth of some fungi was inhibited, providing a favorable growth environment for others.

Another genus that presented great differences between treatments was *Bipolaris*, which was five times less common in conventional plants than in organic plants. Therefore, the application of pesticides appears to induce important changes in the colonizing endophytes.

Some of the genera identified, such as *Acremonium*, *Alternaria*, *Cladosporium*, *Colletotrichum*, *Epicoccum*, *Fusarium*, *Phoma* and *Pestalotiopsis* are considered cosmopolitan and are often reported as endophytic on several species of host plants from temperate and tropical regions (Schulz and Boyle, 2005). The genera *Acremonium*, *Cladosporium*, *Fusarium*, *Paecilomices*, and *Verticillium* were also reported as entomopathogenic fungi; they are able to cause damage via plagues as nematodes, flies, aphids and Lepidoptera, among others (Breen, 1994; Alves, 1998; Robbs and Bittencourt, 1998; Pimentel et al., 2006).

The genus *Alternaria*, besides being often recorded as endophytic, is also considered a phytopathogen in cultures such as mustard (Pedras et al., 2009), tobacco, apples, and tangerine (Tsuge et al., 2012). Therefore, the use of pesticides might favor colonization by potentially pathogenic fungi. Furthermore, some of the toxins produced by this genus present a cytotoxic effect on *Glycine max* plants (Souza et al., 2013). Along with the genus *Fusarium*, *Alternaria* spp. are among the most commonly isolated phytopathogens from soybean worldwide (Roy et al., 2000; Boca et al., 2003; Gally et al., 2006; Broggi et al., 2007). On the other hand, the application of agrochemicals was associated with the incidence of the genus *Chaetomium*, which was isolated only from the conventional crop. *Chaetomium* has been described as endophytic in several host plants including *Gingko biloba* (Qin et al., 2009), grasses (Park et al., 2005) and corn (Pimentel et al., 2016), and is also capable of producing bioactive compounds that might benefit the plant and provide biocontrol activity (Pietro et al., 1992), fungicidal activity (Park et al., 2005), and antimycobacterial activity (Kanokmedhakul et al., 2002).

Diversity, richness, and evenness

Horn's index indicated similarity of 85% between the first and second sampling events for conventional soybean. The isolates from organic soybean presented 71% similarity. The similarity between organic and conventional soybeans was only 53%. Thus, two distinct communities were characterized: one associated with organic soybean and one associated with conventional soybean. Some genera, such as *Aureobasidium*, *Bleptosporium*, *Paecilomyces*, *Pyricularia*, *Trochothecium*, *Cladosporium*, *Rhizoctonia*, *Sclerotium*,

Scopulariopsis, *Scytalidium* and *Stemphylium*, were present only in the organic crop. The higher number of genera isolated from organic soybean, along with the low (53%) similarity between the two farming systems, indicates a microbiotal imbalance in conventional soybean.

Based on Shannon's index and maximum theoretical diversity values, a high diversity of endophytes was associated with organic soybean in both the first and second sampling events (Table 2). This contrasted with the observations in conventional soybean, which presented increased diversity in the second sampling event. However, the diversity values remained lower than the values for the organic crop. Richness and evenness values in the first and second sampling events were also higher in the organic crop than in the conventional crop. The differences in H', R and E, in conventional soybean, observed between the sampling events, may have been due to the size of the leaves and the time of exposure to the different organisms.

This reduction in diversity, richness, and evenness was directly associated with the farming system. Low-diversity environments present a constant imbalance and are subject to the permanent disappearance of species that are important for ecosystem maintenance (Magurran, 1988). Decreased diversity, richness and evenness when organic and conventional practices were compared, were also observed in corn, melon, peppers, and tomato (Xia et al., 2015). This again highlights the imbalance resulting from the use of biocides in conventional management systems, a community with low diversity, with a few dominant genera, such as *Alternaria*, which represented more than half of the isolates obtained from that system. In contrast, the higher diversity, richness, and evenness in the endophyte community isolated from organic soybean indicate a balanced system with a rich community and several genera that are equally abundant.

This work focused on leaf endophytes; however soil microbial communities are often used in modern microbiology to demonstrate biocide toxicity. Hsiao et al. (2013) showed that some benzoylurea-class insecticides may affect soil bacterial biodiversity, besides leaving residues that are toxic to the environment. Other pesticides, such as atrazine, are also able to produce negative impacts on soil bacterial diversity (Chen et al., 2015). It has also been found that the use of fungicides can affect the availability of soil micronutrients (Paul et al., 2013). Some herbicides can impact the symbiotic relationships between plants and mycorrhizae, decreasing fungal sporulation and infectivity (Pasaribu et al., 2013). The same can be seen with insecticides, which may affect the germination of mycorrhizal spores in soybean and decrease its percentage of colonization by those fungi (Menendez et al., 1999).

Triflumuron and fenoxaprop-P-ethyl are considered to have high human toxicity and to be dangerous to the environment (Reference). In spite of their widespread use, the present work appears to be the first to evaluate the undesirable effects of triflumuron and fenoxaprop-P-ethyl on microbial communities associated with different agricultural practices. The present work indicated that the application of triflumuron and fenoxaprop-P-ethyl induced an ecological imbalance in the endophytic fungi microbiota in soybean. However further studies are necessary to evaluate the direct impact of those pesticides on fungal growth. Additionally, the use of pesticides led to decreased diversity, richness and evenness of the microbial community.

Table 2
Diversity, richness, and evenness values in organic and conventional soybean crops, 55 d, and 75 d after seed germination.

Crop management	Diversity		Maximum theoretical diversity		Richness		Evenness	
	55 d	75 d	55 d	75 d	55 d	75 d	55 d	75 d
Organic	2,94	2,81	3,33	3,26	3,61	3,86	0,68	0,64
Conventional	1,02	1,36	2,71	2,83	1,87	2,57	0,18	0,23

Conflict of interest statement

The authors state that there are no conflicts of interests.

Acknowledgments

The Capão da Palmeira Farm provided the seeds and planting sites for the field experiment. Dr. José Marcelo Rocha Aranha (UFPR) helped in the interpretation of the ecological indices and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) provided funding.

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