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# Non-target effects of fungicidal pea (*Pisum sativum* L.) seed treatment on soil microorganisms

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## Abstract

Fungicides are widely used to control pea diseases. We investigated non-target effects of fungicide seed treatment, applied at recommended rates, on soil bacteria and fungi counts in the rhizosphere and bulk soil in Lithuania from 2008 to 2010. Microbiological analyses were conducted on the soil samples of the rhizospheric and non-rhizospheric zones of pea plants, the seeds of which either had previously been dressed with Raxil extra and Kinto or had not been dressed (control plots). Seed treatment exerted a negative effect on the fungal abundance in the pea rhizosphere. Rhizosphere fungi levels were by 40.6–33.4% lower on Raxil extra and Kinto treated pea at seedling stage compared with the untreated. At bud formation stage, the seed-treatment effect on the spread of fungi was lower. Rhizosphere bacteria amount was significantly higher than in the non-rhizosphere. In most cases, fungicide effects were positive for bacteria present both in the rhizosphere and bulk soil throughout the course of the experiment, except for seedling stage in 2010 in a wet soil. The amount of rhizosphere bacteria weakly reacted to fungicide treatment during bud formation stage in 2009, while in 2010 showed a significant positive effect. The fungicide treatment did not have any major effect on the growth of bacteria in the bulk soil during seedling stage and bud formation in both years.

Key words: pea, root rots, seed treatment, rhizosphere, microorganisms.

## Introduction

In a rotation with cereal crops, pea breaks cereal disease cycles, improves soil tilth, and due to symbiotic relationships with soil bacteria *Rhizobium leguminosarum* enhances fertility by fixing atmospheric nitrogen into plant-available forms. Pea is highly susceptible to pre-emergence damping off and after emergence root and foot rots caused by soil borne and seed borne fungal infection (McPhee, 2003). Because cultural control measures are relatively ineffective, effective management against diseases have been achieved by application of fungicides. Effective seed treatment managed reduction of seed borne inoculum and lessened introduction of the pathogens to the new areas (Hwang et al., 1991; Gaurilčikienė et al., 2008). On the other hand, a greater use of agrochemicals causes major problems, such as soil and water pollution and the negative impact caused on the biodiversity because of their ability to also affect non-target species. Soil quality is commonly defined by a number of physical, chemical and biological properties that determine the potential to maintain plant health, and the soil biological productivity (Chellemi, Porter, 2001). All these factors influence plant health as an important factor that limits optimisation of yield and its quality (Cook, 2000). Improvement of soil quality and plant health depends, however, on the cropping system, and overall agricultural practices, e.g., use of pesticides, organic amendments or tillage (Cook, 2000; Chellemi, Porter, 2001).

Rhizosphere is a zone that surrounds the plant roots, and where there are complex relationships between plants, soil organisms and the soil. Rhizosphere of plants accumulates several tens of times more microorganisms than in bulk soil. The greatest biological activity is characteristic of the rhizosphere soil (Lilieroth et al., 1990) due to the role of compounds exudated by the roots of different cultivated plants. Beneficial microorganisms are the first to occur on roots and directly stimulate plant growth, protect the soil from destruction and preserve favourable ecological conditions in the soil (Heckman, Strick, 1996). Diverse microbes have the capacity to inhibit the growth and activities of various soilborne plant pathogens (Whipps, 2001).

Crops largely depend on the phytosanitary condition of the soil. Fungicide for seed treatment, in addition to its primary purpose to reduce seed-borne pathogens can alter the activity of microorganisms and plant growth. Fungicides are nowadays commonly used to control fungi parasitizing on crop plants, including legumes. The indiscriminate using of excessive fertilizers and pesticides for maximum crop production has resulted in a nutrient-sufficient rhizosphere environment leading to less-defensive root environment for pathogens. Such environment has also resulted in changed rhizosphere activity by which the associative plant growth-promoting bacteria remain free in the rhizosphere and the pathogens cause more damage (Parmar, Dufresne, 2011). A portion of the pesticides and fungicides interact with microorganisms in the soil and rhizosphere (Wooton et al., 1993). Pesticides may strongly alter the ecology and the physiology of bacteria and fungi.

A lot of research has been conducted for assessing the dissipation of fungicides in soil and their effects on soil microorganisms (Kinney et al., 2005). Some studies have demonstrated the negative effects of pesticides on microbial survival and growth only in high concentrations (Cycon et al., 2006). It has been previously demonstrated that some pesticides are toxic to *Arbuscular mycorhiza* fungi, reducing sporulation, hyphal growth and root colonization in *Pisum sativum* (Schreiner et al., 1997). Walley et al. (2006) found that chemical pesticides affect nitrogen fixation and nodulation by *Rhizobium* in pulse crops. Pathogenic and non-pathogenic, beneficial and non beneficial fungi as well as bacteria, all were, more or less, affected by chemical pesticide.

Some studies are presented concerning the impact of pre-sowing seed treatment with a chemical preparation on the healthiness of *Phaseolus coccineus* and on the formation of microorganisms in the rhizosphere of this plant (Patkowska, 2009). Most field studies on fungicide or insecticide effects indicate that when they are applied at recommended rates, they usually have no significant effects or have transitory effects on soil microbial characteristics (Ahtiainen et al., 2003). Even in laboratory incubation studies, pesticides usually show significant effects on soil microbial characteristics only at unrealistically high concentrations (Ahtiainen et al., 2003). Nonetheless, results of field studies cannot be generalized because responses depend not only on pesticide properties, but also on soil properties and environmental conditions.

In addition to the fungicides directly inhibiting pathogenic rhizosphere microbes, there exists microbial antagonism phenomenon able to suppress diseases (Estevez de Jensen et al., 2002). Although in this study we did not explore antagonism, but apparently it exists in the soil. The active microorganisms may compete with the soilborne plant pathogens for nutrients, or essential elements or they may produce antifungal compounds (Srivastava, Shalini, 2008).

A lot of information is found in literature on the effect of fungicidal preparations on the disease control, while reports concerning their effect on soil microorganisms directly related to the rhizosphere of legumes are scarce. Information regarding the effects of seed treatment fungicides on non-target soil microorganisms in Lithuania is required, as well as better understanding of soil quality in relation to plant health is also important.

The objective of this study was to estimate the effect of seed treatment, applied at recommended rates, on soil bacteria and fungi amount in the rhizosphere and bulk soil.

## Materials and methods

*Field experiments.* The field research was carried out during the period 2008–2010 at the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry. The soil of the experimental site is light loam *Endocalcari-Endohypogleyic Cambisol* (*CMg-n-w-can*). The main agrochemical parameters of the arable layer: KCL $pH_{KCl}$ 7.0, humus content from 2.1% to 2.3%, and a moderate amount of available soil phosphorus and potassium. Pea (*Pisum sativum* L.) cv. 'Pinochio' was used to assess the impact of fungicide seed treatments on the progress of pea root and foot rot diseases. Kinto (a.i. triticonazole and prochloraz 20 + 60 g $l^{-1}$) 1.5 l $t^{-1}$ and Raxil extra (a.i. tebuconazole and thiram 15 + 500 g $l^{-1}$) 2.0 l $t^{-1}$ were used for pea seed treatment using "Amazone" (Germany) seed treater with the water slurry 10 l $t^{-1}$. An untreated control was used. A split-plot randomized complete block design with four replicates was used. The field trials were sown at a seed rate of 1 million viable seeds per ha with a "Fiona" (Denmark) drill. The plots were 3 m wide and 24 m long with row spacing of 12.5 cm. The grain was harvested at complete maturity with a plot harvester "Wintersteiger Delta" (Germany).

*Rhizosphere and soil analysis.* Microbiological analyses were conducted on the soil samples of the rhizospheric and non-rhizospheric zones of pea plants, the seeds of which either had previously been treated with Raxil extra and Kinto or had not been treated (control plots). At seedling (BBCH 11–12) and bud formation (BBCH 51–55) stages, soil samples were taken from the plots between the rows (0–7.5 cm depth) (recognized as a non-rhizosphere soil) and from soil closely adhering to plant roots (recognized as a rhizosphere soil). Thus prepared samples of rhizosphere and non-rhizosphere soils were used for a serial-dilution plate count technique. A soil sample was suspended, and a series of decimal dilutions was prepared. Aliquots of appropriate dilutions were transferred to Petri dishes in triplicate onto tryptic soy agar ("Liofilchem", Italy) to isolate general heterotrophic bacteria. Total fungal counts were determined on malt extract agar ("Liofilchem", Italy). The plates were incubated at 23°C and bacterial and fungal counts were made after 4 and 7 days, respectively. Plates with 30–300 colony-forming units (CFU) were selected for counting. The CFU were corrected for moisture content of soil before the final counts were expressed as the number of culturable bacterial and fungal cells per 1 g of dry soil.

The disease severity index of foot and root rots (DSI %) was calculated for each plot by the following formula: $DSI = \sum(pn)100 / PN$, were $\sum(pn)$ – sum of the point product with the number of plants affected at this point, P – the highest point value of the scale (5), N – number of plants assessed (Gilpatricc, Bush, 1950).

*Meteorological data.* During the period 2008–2010, metrological conditions varied between years in the central part of Lithuania were the field trials were established. In 2008, the temperature of April and May was 1.5°C higher compared with the long-term average. During the last ten-day period of April and May droughty conditions prevailed and only 25% of the long-term annual precipitation amount fell. In May, hydrothermal coefficient (HTC) was 0.4 (normal – 1.4). The first ten-day period of June was also without rain, and the average temperature of the ten-day period was 2.2°C higher than the long-term average. In the second half of June, heavy rainfall occurred and plant growth conditions improved considerably.

Droughty conditions prevailed also in May 2009. The temperatures during April and May were about 1.5°C higher than the mean. June was very wet, it rained for 12 days in succession, and 2.8 times more rainfall fell compared with the long-term mean. July was hot and also wet and the amount of rainfall was 35% higher than the mean.

In 2010, the spring was warmer and wetter than the long-term mean. In the second ten-day period of May, 40.5 mm of rainfall fell, almost a monthly rate. June's temperature was 0.6°C higher than the mean, rainfall exceeded the mean by 18%. July was very wet; rainfall was nearly twice as high as the long-term mean. Mean monthly temperature was by 4.1°C higher than the mean.

*Statistical analyses.* The data are reported as mean ± standard error of the mean and were examined using analysis of variance (*ANOVA*) procedures. Significant differences among treatment means were assessed by Fisher's least significant difference test (LSD, $p < 0.05$). Statistical computations were performed with software *ANOVA* adapted by Tarakanovas in the *Visual Basic of Application* as macro program to run in the *Excel* (Tarakanovas, Raudonius, 2003). All our experiments were carried out in triplicates.

## Results and discussion

The application of fungicides to control plant diseases has become a common practice in crop production in many parts of the world. However, there is a need to characterize the most suitable and susceptible biological indicators of adverse effects of fungicides on the soil environment. Seeds are considered to be a suitable host to maintain the pathogenic microorganisms even in the absence of the host. Treating such seeds with fungicides or bactericides will protect them from being attacked by fungi, nematodes or other pests (Buss et al., 2001). Treating crop seeds with fungicides will protect them against soil-borne fungi; however, it was found that they have harmful effect on plants and microorganisms. Therefore, there was a crucial need to study their toxicity (Ramadan et al., 1990).

The analysis of pea root disease showed that both at the stage of seedlings and at bud formation the severity index on pea root treated with fungicides was significantly lower than in the control (Table 1). The main pathogens of pea root rots, identified as *Phoma pinodella* that prevailed among the fungus *Ascochyta* complex and *Fusarium* spp., were the most frequently isolated ones from root rot lesions of diseased pea plants.

Seed fungicide treatment not only suppressed the occurrence of pathogens (Table 1), but also had impact on the pea rhizosphere's fungal abundance. Preliminary analysis of the abundance of the pea rhizosphere fungi in 2006 showed that seed treatments by Bariton and Maxim star significantly inhibited pathogens (Gaurilčikienė et al., 2008).

***Table 1.*** Pea root disease severity index (%)

Dotnuva, 2008–2009

| | BBCH 11–12 | BBCH 51–55 | BBCH 11–12 | BBCH 39–40 | BBCH 11–12 | BBCH 39 |
|---|---|---|---|---|---|---|
| | 2008 | | 2009 | | 2010 | |
| Untreated | 20.6 | 30.6 | 3.6 | 32.7 | 0.18 | 40.2 |
| Raxil extra | 8.9 | 6.7 | 0 | 8.7 | 0.04 | 6.3 |
| Kinto | 2.2 | 4.9 | 0 | 5.35 | 0.1 | 19.0 |
| $LSD_{05}$ | 4.46 | 4.94 | 0.78 | 4.23 | 0.06 | 10.02 |

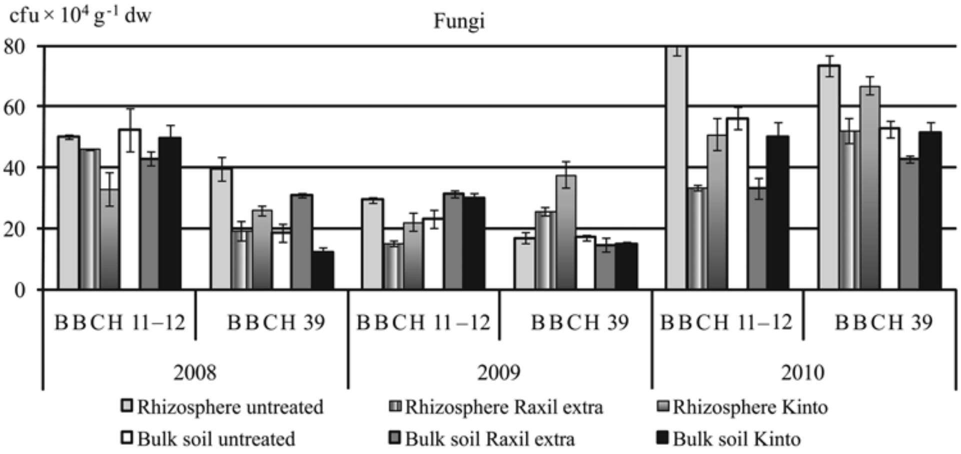
With regard to microbial content in both rhizosphere and non-rhizosphere, the influence of fungicides was less pronounced and inconsistent after a longer time after seed treatment. Seed treatment markedly decreased the number of fungal colonies in general, as shown in Figure 1. The mycoflora of the rhizosphere was considerably inhibited by both fungicides (Table 2).

***Table 2.*** Results of two-way *ANOVA* (F-values and significance) for fungi

| Source of variation | F actual | df | *P* | F actual | df | *P* |
|---|---|---|---|---|---|---|
| | BBCH 11–12 | | | BBCH 39 | | |
| 2008 | | | | | | |
| Site (S) | 1.98 | 1 | 0.189 | 13.13** | 1 | 0.004 |
| Treatment (T) | 2.45 | 2 | 0.136 | 7.64** | 2 | 0.009 |
| S × T | 2.5 | 2 | 0.132 | 21.22** | 2 | 0.0002 |
| 2009 | | | | | | |
| Site (S) | 12.94** | 1 | 0.0048 | 31.49** | 1 | 0.0002 |
| Treatment (T) | 1.39 | 2 | 0.292 | 7.5** | 2 | 0.01 |
| S × T | 15.58** | 2 | 0.0008 | 11.02** | 2 | 0.0029 |
| 2010 | | | | | | |
| Site (S) | 6.11** | 1 | 0.033 | 74.1** | 1 | 0.0000 |
| Treatment (T) | 36.86** | 2 | 0.00002 | 28.28** | 2 | 0.0000 |
| S × T | 5.42** | 2 | 0.0254 | 3.34* | 2 | 0.077 |

*, ** – significant at the 0.05 and 0.01 probability levels, respectively

The seed treatment effect on the amount of fungi at seedling stage during the three experimental years showed the same trend – there was identified 41–33% less fungi than in the untreated (Fig. 1). At seedling stage, the use of Raxil extra and Kinto decreased fungi population (46.1 × $10^4$ cfu and 32.9 × $10^4$ cfu, respectively) as compared with the untreated (50.1 × $10^4$ cfu) in 2008. In 2009, the amount of fungi decreased by 33.8% and 24.5%, in 2010 by 58.2% and 36.1%, for Raxil extra and Kinto, respectively. There was detected negative fungicide impact on the amount of fungi in bulk soil during seedling stage, except for the year 2009. In 2008, the fungi amount in bulk soil in average for both treatments decreased by 11.6% and in 2010 by 25.8%. This is in agreement with the findings of other authors who reported that the total population of fungi of the soil after the application of some fungicides was significantly lower than in the control (Patkowska, 2005; 2009).



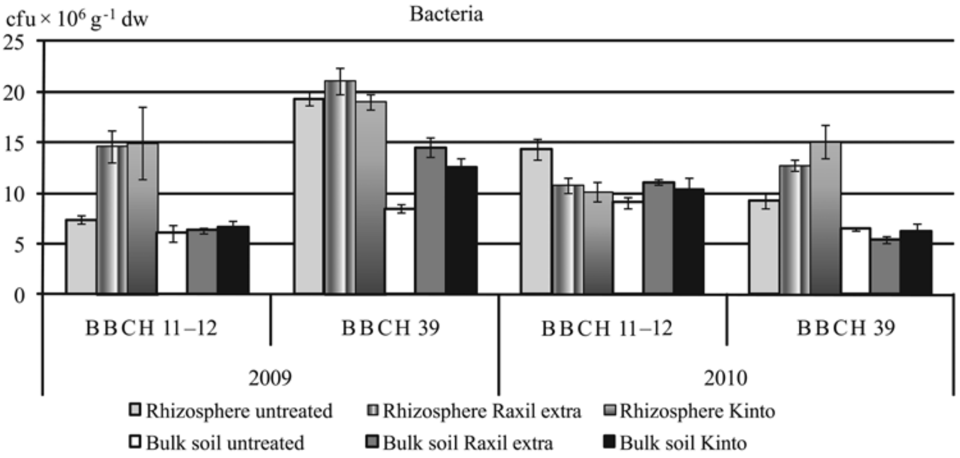
Means ± standard deviation

***Figure 1.*** The influence of fungicide treatment of seeds on the total number of fungi ($10^3$ g$^{-1}$ dry wt) in the rhizosphere and bulk soil

At the bud formation stage, the trend remained similar, there was detected less fungi on the rhizosphere of treated plants. The fungicide treatment of seeds decreased the amount of fungi by 42.9% and 19.3% in 2008 and 2010, respectively. However, in 2009 the impact of fungicide treatment was the opposite, the treated pea rhizosphere contained higher counts of fungi than the untreated. Such differences in the amount of fungi may have resulted from the differences in weather conditions, when 2009 was extremely dry, and 2010 extremely wet.

Fungicides did not result in consistent changes in the abundance of fungi in the soil between the pea plants at bud formation stage. The population of fungi at the bud formation stage may be either increased or decreased, since the pesticide effect vanished, and the differences revealed were accidental. Raxil extra exhibited a stronger negative impact on the amount of fungi compared with Kinto.

Seed treatment with fungicides had impact on the amount of the rhizosphere culturable bacteria community (Table 3). The development of bacteria was assayed for two years. The changes in bacteria population on the rhizosphere and bulk soil are shown in Figure 2. Abundance of bacteria in the rhizosphere and non-rhizosphere soil of pea plants was ambiguous between years and in many cases was opposite to the amount of fungi. Some studies have shown that fungicides reduce fungal biomass or counts, but have a positive effect on the communities of bacteria in the rhizosphere (Monkiedje et al., 2002; Patkowska, 2005; 2009). The biomass or population of bacteria increases because bacteria use the fungicide (during degradation) and fungal necromass (i.e. dead fungi) as carbon and nutrient sources for energy and growth. Actually, in our study in 2009 there was found lower amount of fungi, but higher amount of bacteria in rhizosphere soil during the seedling stage. Conversely, the deleterious effect of seed treatment on rhizosphere bacteria during seedling stage was proved in 2010 of this research. There was detected positive impact ($P < 0.05$) of fungicides on pea rhizosphere during seedling stage in 2009 (Table 3). Our results are in agreement with the study of Durska (2003) who noted that fungicide stimulated the growth of bacteria except for the periods of budding. However, significantly negative influence of fungicide seed treatment on bacteria amount in rhizosphere was detected in 2010 at seedling stage. The negative fungicide effect also was observed in plants whose seeds were treated in other studies (Correa et al., 2009).



Means ± standard error

***Figure 2.*** The influence of fungicide treatment of seeds on the total number of bacteria ($10^6$ g$^{-1}$ dry wt) in the rhizosphere and bulk soil

***Table 3.*** Results of two-way *ANOVA* (F-values and significance) for bacteria

| Source of variation | F actual | df | *P* | F actual | df | *P* |
|---|---|---|---|---|---|---|
| | BBCH 11–12 | | | BBCH 39 | | |
| 2009 | | | | | | |
| Site (S) | 20.3** | 1 | 0.0011 | 111.88** | 1 | 0.0000 |
| Treatment (T) | 3.84* | 2 | 0.0579 | 9.08** | 2 | 0.0056 |
| S × T | 3.09* | 2 | 0.0903 | 3.63* | 2 | 0.0651 |
| 2010 | | | | | | |
| Site (S) | 6.49** | 1 | 0.0289 | 105.28** | 1 | 0.0000 |
| Treatment (T) | 1.86 | 2 | 0.2063 | 6.7** | 2 | 0.0142 |
| S × T | 8.63** | 2 | 0.006 | 8.73** | 2 | 0.0064 |

*, ** – significant at the 0.05 and 0.01 probability levels, respectively

The amount of rhizosphere bacteria weakly reacted to fungicide treatment during bud formation stage in 2009, while in 2010 showed a significant positive effect. Over time the plant develops, changes occur in root metabolism and interactions between plants and microorganisms, also in the amount of microorganisms in the soil and rhizosphere. The fungicide treatment did not have any major effect on the growth of bacteria in the bulk soil during seedling stage and bud formation in both years. The bulk soil research showed that the fungicide treatment had only slight positive impact on bacterial abundance at seedling stage. Some clear positive impact on bacteria in bulk soil was noted in 2009 during bud formation stage. It was found that in bulk soil the fungicide treatment of seeds did not significantly change the mean number of the studied bacteria; however, contributed to the proliferation of these microorganisms in the rhizosphere zone (Kaszubiak, Durska, 2000). And this is understandable, since the rhizosphere is relatively rich in nutrients, because as much as 40% of plant photosynthetic products are exudates from roots (Bais et al., 2006), which explains why the rhizosphere bacteria amount is significantly ($P <$ 0.05) higher than in bulk soil.

The formation of microorganism communities in the rhizosphere is extremely important because they affect health and hence the yield of plants.

Most of the field studies on the effects of fungicides indicate that when they are applied as a seed dresser at recommended rates, they usually have no significant effects or have transitory effects on soil microbial characteristics (Ahtiainen et al., 2003). However, some authors showed that pesticide significantly decreased the number of culturable heterotrophic bacteria (El Fantroussi et al., 1999), which agreed with our results. Some of the pesticides had no impact, and some had long-lasting impact on soil bacterial diversity (Ibekwe et al., 2001). Conversely, seed treatment had positive impact on bacteria development (Patkowska, 2005), which also agrees with our study.

## Conclusion

Seed treatment negatively affected the fungi abundance on pea rhizosphere. The amount of fungi on the rhizosphere at seedling stage of pea treated with Raxil extra and Kinto was 57–89% lower compared with the untreated. At bud formation stage, the seed-treatment effect on fungi amount was lower. Fungicide seed treatment did not significantly affect the abundance of fungi in the bulk soil between the pea plants. Most bacteria were found on the rhizosphere. Basically, most bacteria were found on the roots of plants, whose seeds had been treated with fungicides. In most cases, Raxil extra exerted a strong influence on fungi and had no consistent effect on the abundance of bacteria. In a few cases the fungicide effect persisted at later stages of pea growth.

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# Sėjamojo žirnio (*Pisum sativum* L.) sėklos beicavimo fungicidais poveikis dirvožemio mikroorganizmams

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[2]Lietuvos agrarinių ir miškų mokslų centro Perlojos bandymų stotis

## Santrauka

Fungicidai yra plačiai naudojami siekiant apsaugoti žirnius nuo ligų. Tirta sėjamojo žirnio sėklos beicavimo pagal rekomenduojamas fungicidų normas įtaka rizosferos ir dirvožemio bakterijų bei grybų gausumui Lietuvoje 2008–2010 m. Mikrobiologiniai tyrimai atlikti dirvožemio ir žirnių rizosferos mėginiuose daigų bei butonizacijos tarpsniais. Žirnių sėkla beicuota preparatais Raxil extra ir Kinto arba nebuvo beicuota. Sėklos apdorojimas neigiamai paveikė grybų paplitimą žirnių rizosferoje. Daigų tarpsniu rizosferos grybų kiekis buvo 40,6–33,4 % mažesnis ant augalų, kurių sėklos buvo apdorotos Raxil extra ir Kinto. Butonizacijos tarpsniu sėklos beicavimas grybų plitimui turėjo mažesnę įtaką. Dėl beicavimo dirvožemyje nebuvo nustatyta esminių grybų gausumo pokyčių. Daugeliu atvejų fungicidų poveikis bakterijų plitimui ir rizosferoje, ir dirvožemyje buvo teigiamas viso tyrimo metu, išskyrus žirniams esant daigų tarpsnio 2010 m. Fungicidų poveikis rizosferos bakterijoms butonizacijos tarpsniu buvo silpnas 2009 m., o 2010 m. nustatyta esminė teigiama beicavimo įtaka bakterijų plitimui. Fungicidai viso tyrimo metu neturėjo didelės įtakos dirvožemio bakterijų kiekiui daigų ir butonizacijos tarpsniais.

Reikšminiai žodžiai: sėjamasis žirnis, šaknų puviniai, sėklos beicavimas, rizosfera, mikroorganizmai.