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EFFICACY OF *Trichoderma harzianum* AND *Bacillus subtilis* AS SEED AND VEGETATION APPLICATION COMBINED WITH INTEGRATED AGROECOLOGY MEASURES ON MAIZE

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ABSTRACT

In the context of sustainable practices and European strategy to reduce the chemical inputs in agricultural conventional systems, on field crops there are approached agroecological practices that targets the performance growing through effective measures that adjust the biodiversity of agroecosystems. The study presents the results of a field experience developed in a farmer participative approach on two years (2021, 2022). In his farm - from Buești, Ialomița, Romania - were established 30 multifactorial testing varieties composed from treatment with a special product for maize crop (BioZeaFert: BF) from *Bacillus subtilis* ICCF 84: 25%; *Bacillus subtilis* 284: 25%; *Trichoderma harzianum* ICCF 179: 50%. The treatments have been applied on seeds (Untreated, BF 10%, BF 20%, BF 30%, $CuSO_4$ 10%) and on vegetation period (Untreated, BF 75 g/ha, BF 112.5 g/ha, BF 225 g/ha, $CuSO_4$ 300g/ha) for 3 maize hybrids (Iezer, Felix, F 423). Also has been integrated in his crop technology cover crops and the using of organic agriculture inputs. The application of the product and agroecological measures had positive effects regarding the protection against pest *Tanymecus dilaticollis*. The damage degree was 16-19% in the untreated maize, while for the treated maize it was between 6-11%, the lowest values were observed for BF 30% on Iezer hybrid. The biostimulator had positive effects on plant growth and therefore on yield. The treatments or the type of hybrids did not influenced the weeding degree.

Keywords: *Trichoderma,* on-farm research, agroecology, participative, cover crop.

INTRODUCTION

Agroecology it is known as a complex, three side concept that involves science, agricultural practices and social movement. Regarding science part it is open to participatory approaches and a holistic knowledge, as a social movement encourage small farmers, family farming, resilience of food systems by strengthening farmer independence on agricultural inputs (Europe agroecology - A European association for agroecology, 11.11.2022). There are several steps for transforming a conventional system into a fully agroecological one at a farm level. The first stage or level is to ”*increase the efficiency of industrial and conventional practices in order to reduce the use and consumption of costly, scarce, or environmentally damaging inputs*” (Gliessman, 2016). The second step is to replace the conventional inputs with nature-based solutions, preferably internal produced. At this level, plant diseases and pests are managed by natural (obtained by non-chemical synthesis) plant protection products and several practices for soil quality are performed in order to replace the nitrogen chemical inputs. This practices, besides using natural replacements are involving: cover crops, organic compost, living mulching and a better soil management.

Maize is one of the most important crops worldwide and in Romania hold a top position

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among cultivated cereals. Usually if we analyse the surface of total cultivated cereals in Romania, maize area overcomes 50% (Dragomir et al., 2022). In the last decays, the maize cultivars and cultivation practices, also due to the development of specialised chemical weeds control by herbicides (Şerban et al., 2021) has adapted to fully mechanized operations. For the alleviation of intensive technologies, the principle of covered soil, that states to use cover crops or living mulching, it is an approach recommended by different sustainable agriculture systems (Oberč and Schnell, 2020): agroecology, conservation agriculture (Cizmaș and Cociu, 2015), regenerative agriculture, etc. Cover crops mixtures sequestrate carbon, improve physical soil properties, reduce erosion and retain nitrogen in soil upper layers (Petcu et al., 2022).

For stable yields it is necessary a good management of soil quality inside a farm. Soil fertility, often defined in relation with the specific crop, is a complex of physical, chemical and biological parameters (Petcu et al., 2014). Living organisms applied on seeds or on plants improve the microbiome that is protecting plant against soil and seed pathogens, a major problem on cereal health (Zaharia et al., 2022). Also the seeds microorganisms improves the roots capacity for nutrient absorption (Glick, 2020).

Trichoderma harzianum it is among beneficial fungi used as a seed and soil treatment to control root diseases on various field crops (Alexandru et al., 2013). Different strains and product formulations register different efficacy in controlling fusarium or charcoal rots diseases (Foroutan, 2013; Orojnia et al., 2021).

Bacillus subtilis is a Gram positive bacteria that colonize the plant roots and stimulate the growth of plants (Sicuia et al., 2015).

Trichoderma and *Bacillus* has a wide biodiversity of species and strains with major role in soil fertility (Rey et al., 2004; Gurikar et al., 2022).

Therefore, previous studies have confirmed the effectiveness of *Trichoderma harzianumin* in controlling fusarium or some rots diseases and Bacillus subtilis for improving soil fertility, but this study is among the first report, that have explored the usage of combination between *Trichoderma harzianumin* and *Bacillus subtilis* in managing the yield of maize under field conditions.

MATERIAL AND METHODS

A two years (2021 and 2022) complex field experience was established in a participatory, on farm research manner. An entire field of 3,26 ha - located in Buești, Ialomița County, Romania - was involved for changing farmer's agricultural system and testing the biological efficacy of different seed and vegetation treatments. The GPS coordinates of the fields are: 44°31'59.88"N; 27°10'22.64"E.

It has been used three different maize hybrids of high genetical value from 401-500 FAO group: Iezer, Felix and F 423. The hybrids has proven in previous years stability, high yields potential and medium resistance to *Fusarium* pathogens in different locations of Romania (Horhocea et al., 2020). The effective seed quality results (germination - %, purity - %, thousand kernel weight - g, the presence of pathogens and pests) of the seeding used material are presented in Table 1.

Table 1. Seed quality results of used material

Maize hybrid	Gemination (2021)	Gemination (2022)	Purity (2021, 2022)	TKW (g) 2021	TKW (g) 2022	Pathogens and pests
Iezer	90%	97%	99.9%	221.0	283.3	0
Felix	95%	99%	99.9%	258.9	295.6	0
F 423	97%	100%	99.9%	274.9	280.9	0

All three maize cultivars were treated on seed and in vegetation period with a formulated product, BioZeaFert (with active ingredients *Trichoderma harzianum* strain ICCF 84, 50%, and *Bacillus subtilis* strain ICCF 84, 25%, *Bacillus subtilis* ICCF strain ICCF 284, 25%, plus the pullulan polysaccharide as adjuvant, with excellent film-forming abilities and adhesive properties non active ingredient 0.2%) in 3 different doses (for seed treatment 1 kg/t, 2 kg/t, 3 kg/t, representing concentrations of 10%, 20% and 30%. As a reference product, it was used Copper Sulphate in concertation of 10%, product that is also accepted in organic agriculture. Therefore, it was established at the beginning an experimental design with 15 variants of two factors: 3 maize hybrids and 5 seed treatments (untreated, 3 dosages of BioZeaFert and the reference product) with 5 replicates only for seed treatments and also 5 replicates for both treatments: seed+vegetation. A diagram of the farm participatory research field is shown in Figure 1.

The seed treatment was performed in the previous day before seeding time. In vegetation period, the dosage for the BioZeaFert product were 75 g/ha, 112.5 g/ha, 225 g/ha, and 300 g/ha for $CuSO_4$. The dosages are depicted in Table 2. The vegetation treatment was applied just on half of the field. The design is a trade-off between the need of a field experience for data and the farm conditions equipment to perform the experiment.

Table 2. The testing items dosages on seeds and vegetation period

Applied treatments on seed		BioZeaFert (Dose 1)	BioZeaFert (Dose 2)	BioZeaFert (Dose 3)	$CuSO_4$
Application dose on seed kg/t	*(kg)*	1	2	3	1
Water volume/t	*(l)*	10	10	10	10
Concentration	*(%)*	10	20	30	10
Applied treatments in vegetation period		BioZeaFert (Dose 1)	BioZeaFert (Dose 2)	BioZeaFert (Dose 3)	$CuSO_4$
Application dose on seed g/ha	*(g)*	75	112.5	225	300
Water volume/ha	*(l)*	300	300	300	300
Concentration	*(%)*	0.25	0.38	0.75	1

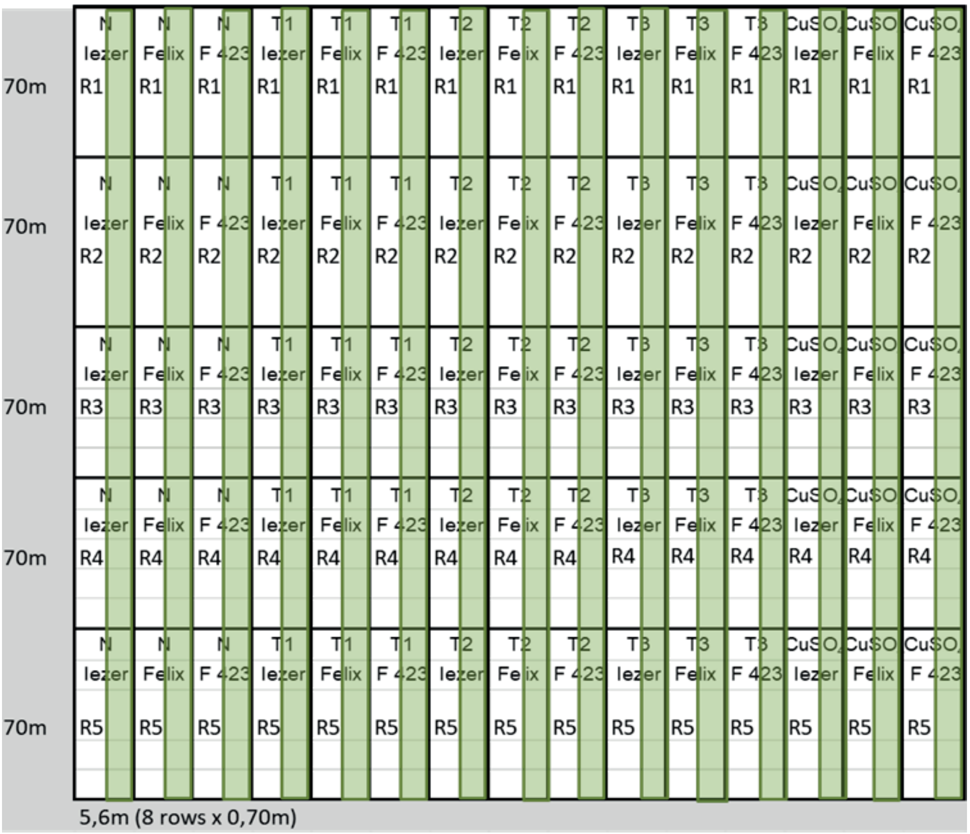

Figure 1. Diagram of on-farm participatory research field, Buești, Ialomița
(*vegetation treatment is represented with green strips, the white areas are just seed treatments*)

Soil type from Bueşti is Levigate Chernozem, clay textured, with pH values slightly acid and over 2% of humus content. During experimentation years a series of soil analyses had been performed in order to follow up the soil improvement characteristics. The soil analysed samples were compound from 25 points, from 0-20 cm soil depth, in conformity with standard STAS 7184/1-84. The pH was measured with a pH meter in water-soil solution (1:2.5 soil/water proportion). For Nitrogen determination was used Kjeldahl method. Organic content was determined by Walkey - Black method, modified by Gogoaşă, the phosphorus content was determined after Ègner-Riehm-Domingo method using ammonium acetate-lactate at pH = 3.7 determined by colorimetry with molybdenum blue (Borlan and Răuţă, 1981; Mărin et al., 2021). The values are presented in Table 5 from the Results and Discussion section.

Before the first year of trials, in 2020, was applied an intensive conventional technology. In autumn of 2020 was applied 30 t/hectare of manure. The main soil tillage was ploughing at 25 cm depth. In 2021, the soil was mobilised with a disk and before seeding was finely prepared for seeding with a spring cultivator. A record of main agricultural and experimental operation for 2021 and 2022 is presented in Table 3.

Table 3. Main agricultural and on-farm participatory research activity record of years 2021 and 2022

Date (dd.mm.yyyy)		Agricultural/Experimental operation
13.04.2021	13.04.2022	Seed tretment
-	15.04.2022	Ploughing
14.04.2021	16.04.2022	Disking
-	16.04.2022	Base Fertilisation - 35% P_2O_5 BioPhos - 620 kg/ha
14.04.2021	16.04.2022	Seedbed preparation
14.04.2021	16.04.2022	Plotting and Seeding
28.04.2021	30.04.2022	Mechanical weeding
12.05.2021	16.05.2022	Crop density, weed cover and pests assessment
12.06.2021	15.06.2022	Plant vigour, vegetation stage assessment
15.06.2021	18.06.2022	Mechanical weeding
08.07.2021	09.07.2022	Vegetation treatment
10.07.2021	10.07.2022	Mechanical weeding
07.08.2021	10.08.2022	Plant high, cob high assessment
15.09.2021	05.09.2022	Harvesting and samples; Assessment
15.09.2021	06.09.2022	Soil Tillage
16.09.2021	06.09.2022	Cover crop seeding

The cover crop species have been chosen with farmer option to be a mix of wheat and flax in order to use the advantages of cover crops with fibrous roots.

The weather data for 2021 has acquired from OpenWeatherMap.org and from 2022 an Enten Systems field weather station was installed at farmer's headquarter, about 50 m distance from the field.

The caried observations have been adapted for farming participatory evaluation. It was assessed the plant density of plots, a broad plant attack frequency of pests, especially the maize leaf weevil (*Tanymecus dillaticolis*), one of the most dangerous pests for maize in Romania (Georgescu et al., 2021). Also the weeds distribution, as percent on the plot and the species encountered (Ionescu et al., 2021). Plant height, ear cob high were measured at physiological maturity of the plant. The yield is calculated at humidity level of 14%.

RESULTS AND DISCUSSION

The years of experimentation were totally different from the viewpoint of quantity and monthly reparations of rainfall. In 2022 year, from sowing to physiological maturity stage the cumulated rainfall was of 104 mm, insufficient quantity for covering the maize water supply (Table 4).

Table 4. Average temperature (°C) and monthly distribution of rainfall (mm) during the maize vegetation period. Buiești, 2021-2022

Month	IV	V	VI	VII	VIII	Average	Sum
Temperature 2021	9.44	16.94	20.60	24.81	24.55	19.27	
Temperature 2022	13.34	18.66	23.05	25.31	25.85	21.24	
Multianual average	*11.10*	*17.05*	*20.00*	*23.12*	*22.00*	*18.65*	
Rainfall 2021	38.03	58.97	157.3	37.72	14.9		306.92
Rainfall 2022	12.4	28.4	26.6	18.4	18.2		104
Multianual average	*45.29*	*62.11*	*74.00*	*70.59*	*49.00*		*300.99*

The analysed soil samples, from the first 20 cm of soil layer shows a heterogenous soil, in the second year due to applied organic fertilizers and integrated soil management practices: cover crops and soil tillage (Table 5). Also, together with fertilization, the climate change is indirectly influencing the soil properties (Partal et al., 2021). Due to the short period of experimentation, just two years, it is difficult to state final conclusions about the soil chemical properties.

Table 5. Soil main agrochemical parameters in the farm, Buești, Ialomița

Year	Soil pH	Humus content	Total Nitrogen (Nt)	Mobile Phosphorus (P_{AL})	Mobile Potassium (K_{AL})
	pH units	(%)	(%)	mg/kg	mg/kg
2021	6.91	2.72	0.146	11	143
	6.87	2.66	0.139	8	145
2022	6.05	2.12	0.141	25	159
	6.73	2.23	0.124	31	172

The impact of treatments with *Trichoderma harzianum* and *Bacillus subtilis* on plant growth was evaluated by measuring height of maize at physiological maturity stage. This parameter was positively influenced by the treatments performed in both years of experimentation and an effect of the hybrid and the hybrid x treatment interaction was also highlighted in the conditions of 2022 (Table 6).

The largest effect on plants was obtained with the 30% BioZeaFert that showed the greatest plant height (Figure 2).

Table 6. Analysis of variance for height of maize plants

Source of variance	LD	Mean square	F factor and significance	Mean square	F factor and significance
		2021		**2022**	
Factor A (Hybrid)	2	63	3.01 ns	91	5.28*
Error A	8	20.92		17.25	
Factor B (Treatments)	4	3354.5	83.95***	2176.3	94.97***
AxB	8	29.55	0.73 ns	94.33	4.12*
Error B	48	39.95		22.92	

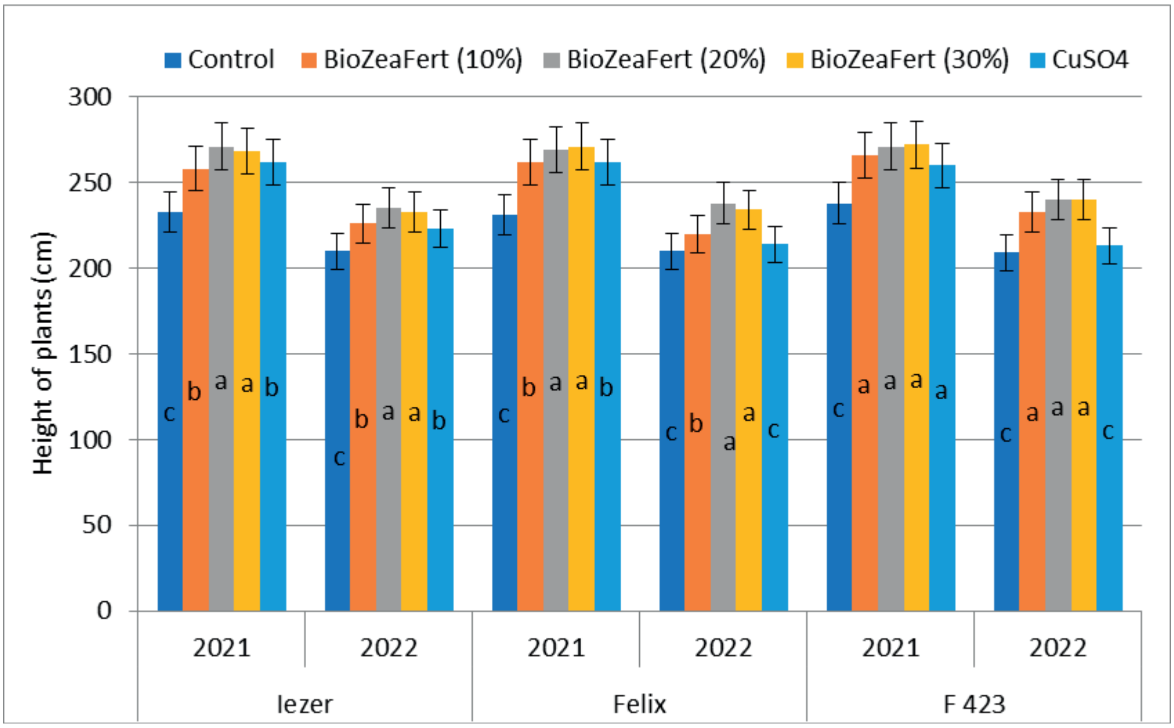

Figure 2. The effect of different treatments on height of maize plants. Error bars indicate standard deviation and different letters indicate significant differences by Duncan's test (p<0.05 at a significance of 0.05).

Research conducted on other plant species has shown that only treatment with the *Trichoderma harzianum* (strain SQR-T037) during P-deficient hydroponic conditions adversely affected the tomato plant growth with a 82% biomass reduction due to the competition between *Trichoderma* and plants for the scarcely available nutrients (Li et al., 2015), but this negative effect was attenuated by combined *Trichoderma*/compost treatments. Our results indicate that the positive synergic action played by the combination between *Trichoderma* and *Bacillus*, probably due to the *Bacillus subtilis* role for stimulate the growth of plants.

The results of the analysis of variance showed that maize yield was very significantly affected by treatments in both years of experimentations and by hybrid only in 2021. The interactions between hybrid and treatments had also significant, respectively, very significant effect on yield (Table 7).

Table 7. Analysis of variance for yield

Source of variance	LD	Mean square	F factor and significance	Mean square	F factor and significance
		2021		**2022**	
Factor A (Hybrid)	2	2318495	52.05***	215845	0.71 ns*
Error A	8	44547		304525	
Factor B (Treatments)	4	3365773	72.52***	2966474	15.09***
AxB	8	362760	7.82***	1209388	6.15***
Error B	48	46409		163574	

The rainfall deficits in 2022 created unfavorable conditions during reproductive organs appearance and grain formations, determining the obtainment of relatively small yields of 4431 kg/ha (average for variants without treatments) - 5402 kg/ha (average for variants with BioZeaFert treatments) (Figure 3).

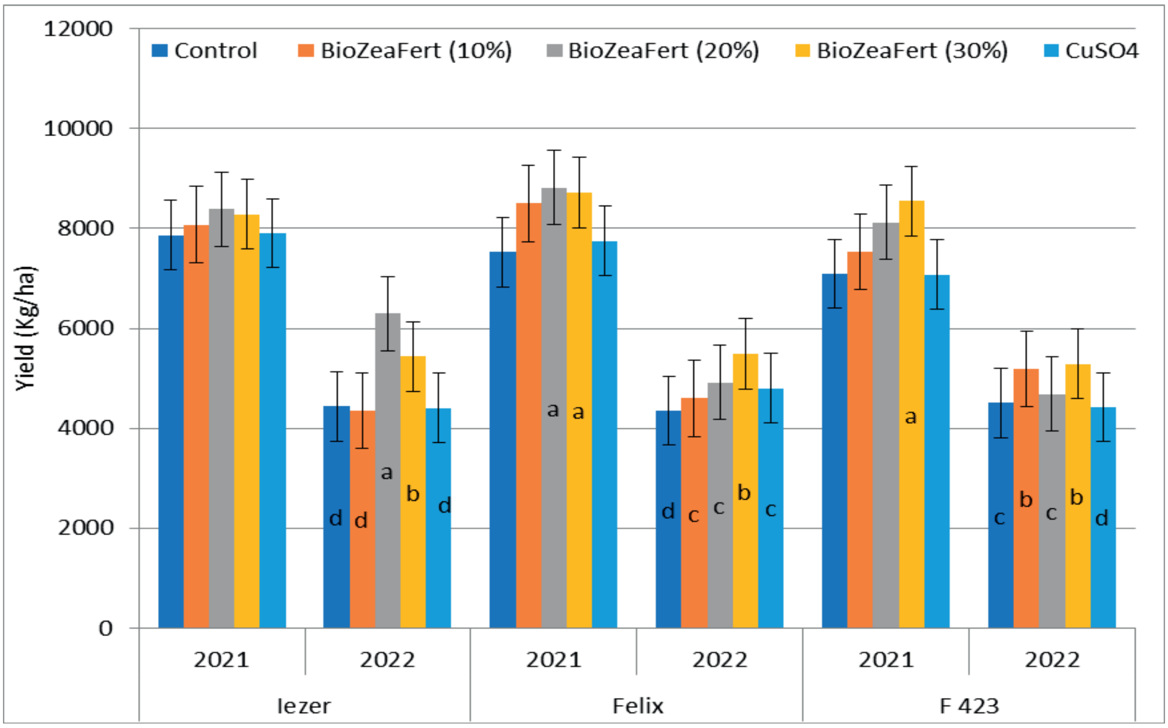

Figure 3. The effect of different treatments on maize yield. Error bars indicate standard deviation and different letters indicate significant differences by Duncan's test ($p<0.05$ at a significance of 0.05).

The increase in production due to the treatments carried out was on average 706 kg/ha (in 2021) and 837 kg/ha (in 2022), the highest values being achieved at the concentration of 30% in both years of experimentation.

Our results are in line with other studies have showed that the application of *Trichoderma harzianum* - *Bacillus subtilis* cell suspensions in the field has achieved synergistic increases in plant growth, yield, and quality of a wide variety of crops, including vegetables, legumes, and fruit trees (Firdu et al., 2020).

The results of the analysis of variance showed that number of maize leaf weevil (*Tanymecus dillaticolis*) was very significantly affected by treatments in both years of experimentations and by hybrid only in 2021. The interactions between hybrid and treatments had also very significant effect only in the year in which water stress was lower (2021) (Table 8).

Table 8. Analysis of variance for maize leaf weevil (*Tanymecus dillaticolis*)

Source of variance	LD	F factor and significance	
		2021	**2022**
Factor A (Hybrid)	2	22.19***	0.52 ns
Error A	8		
Factor B (Treatments)	4	58.94***	10.76***
AxB	8	4.03***	1.76 ns
Error B	48		

We noticed differences in the reduction of the number of *Tanymecus dillaticolis* as a result of the treatments based on *Trichoderma harzianum* and *Bacillus subtilis* more evident in variants with a higher concentration of product (Table 9).

Table 9. The effect of different treatments on the maize leaf weevil (*Tanymecus dillaticolis*) (nr/sm)

Hybrid	Variants	2021	2022
Iezer	Untreated	19 e	40 b
Iezer	BioZeaFert (10%)	9 b	30 b
Iezer	BioZeaFert (20%)	11 c	27 b
Iezer	BioZeaFert (30%)	6 a	21 b
Iezer	$CuSO_4$	9 b	30 b
Felix	Untreated	16 d	41 b
Felix	BioZeaFert (10%)	10 c	29 b
Felix	BioZeaFert (20%)	8 b	25 b
Felix	BioZeaFert (30%)	10 c	19 a
Felix	$CuSO_4$	15 d	28 b
F 423	Untreated	16 d	21 b
F 423	BioZeaFert (10%)	9 b	14 a
F 423	BioZeaFert (20%)	9 b	9 a
F 423	BioZeaFert (30%)	8 b	8 a
F 423	$CuSO_4$	18 e	23 a

The letters indicate significant differences by Duncan's test ($p<0.05$ at a significance of 0.05).

Although, there are few references related to the reduction of the *Tanymecus dillaticolis* attack in maize, it is known below- and above-ground plant-insect-microorganism interactions are very complex and may be very different under field conditions. Contreras-Cornejo et al. (2021) showed that, in a maize field, the community of native foliage arthropods could be altered after plant inoculation with *Trichoderma harzianum* strain 38. In the study, no negative effect on beneficial organism or biodiversity has been observed. Also, there has been no phytotoxic or side-effects of the applied product.

The treatment or different hybrid, had no influence on different weeds spectrum or density between different plots.

CONCLUSIONS

The participatory on farm research trial during two years revealed the biological efficacy of *Trichoderma harzianum* strain 179 combined with *Bacillus subtilis* strains 84 and 284.

The product formulated with active ingredients on living organisms and has no harmful effect on maize crop or other beneficial organisms.

There was an yield gain of seed and vegetation treated plots in typical conditions for area, in 2021 and even bigger in 2022, a difficult year for maize, due to the small precipitation quantity.

The tested microorganisms treatments on seed and vegetation stimulated the plant growth and protection and revealed indirect benefits due to improvement of seed and soil microbionta.

Also, the beneficial observed results in reduction of maize weevil attack and yield gain are due to the complex influence of different agricultural practices which farmer started to implement on his field: changing the base fertilization only to organic inputs and cover crops.

The transition to agroecology practices and organic inputs, adopted in collaboration with research organisations, increased the sustainability of the farm.

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