Revista Mexicana de Ciencias Agrícolas

DOI: https://doi.org/10.29312/remexca.v16i3.3641

Article

Assessment of high dilutions on soil microbial respiration

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Abstract

Soil health and fertility determine its potential to support crop productive capacity. Organic matter is considered an indicator of biostructure and maintenance of life in the soil. Its role is reflected in microbial activity that can be measured by evaluating CO_2 released as a function of metabolic oxidation activities. Microbial respiration, which is more sensitive than physicochemical indicators, provides information on other disturbances. Therefore, the soil microbiota has a high potential to respond to subtle interventions with the application of dynamized high dilutions (homeopathies). Dynamized high dilutions have significantly impacted living things and plant cultivation. This study assessed variations in microbial respiration following the application of these dilutions in Fraiburgo, SC, Brazil, in 2020. Four treatments were used in the laboratory experiments: distilled water (i), 30% alcohol (ii), *Calcarea carbonica* 30CH (iii) and *Silicea terra* 30CH (iv), in 10 replications formed by two independent soil samples. The treatments were applied with a manual sprayer in a ratio of 1:99 of water in three different seasons in the soil. The results showed a significant increase in the rates of CO_2 released by the soil due to the application of high dilutions by the Anova test, $p< 0.05$, especially in the first 48 h. The study showed that dynamized dilutions alter the respiratory dynamics of soil microorganisms.

Keywords:

dynamized dilutions, microbial respiration, soil health.

DOI: https://doi.org/10.29312/remexca.v16i3.3641

Introduction

Soil health and quality can be assessed by physical, chemical, and biological characteristics through indicators that reflect plant development throughout their vegetative-reproductive cycle (Cherubin *et al*., 2015). The carbon stored in the soil is two to three times greater than that released into the atmosphere (Coelho, 2005).

Organic matter is fundamental for the biostructure, productivity, and life in the soil (Primavesi, 1982). Microbial activity, key to soil quality, is measured by evaluating the CO_2 released by the metabolic activities of organic matter oxidation (Medeiros *et al*., 2019). This CO_2 is released into the atmosphere, contributing about 50% of the carbon in the total respiration of the ecosystem (Da Silva *et al*., 2017).

The rate of soil microbial respiration, or basal respiration, has been shown to be more sensitive than physical and chemical indicators for detecting disturbances in the soil (Zhou *et al*., 2020). For instance, nutrient flow can negatively affect these rates (Kaschuk *et al*., 2011). On the other hand, adding organic matter generates changes in microbial respiration due to the rapid growth and greater mineralization of microorganisms (Primavesi, 1982).

The evolution of CO_2 in the soil is therefore a measure of the total biological activity of the soil (Anderson, 1982). Given the sensitivity of microbial respiration analysis (basal respiration), it has the potential to respond to subtle interventions, such as the application of dynamized high dilutions. The action on microorganisms was reported by Trebbi *et al*. (2016) in the *in vitro* germination of *A. brassicicola* spores.

Dynamized high dilutions are regulated for organic food production in Brazil and have shown excellent results in agriculture in several countries (Brazil, 2014; Domingues *et al*., 2019; Campos and Pedroso, 2020). According to Bell *et al*. (2002), these dilutions produce nonlinear responses in living organisms, where the inputs are disproportionate to the outputs obtained.

In agriculture, this can translate into higher productivity (Kaschuk *et al*., 2011; Bellavite *et al*., 2014; Domingues *et al*., 2019; Oliveira *et al*., 2020a). The respiration rate, as a variable sensitive to disturbances, can reflect the action of these dilutions on soil microorganisms. This work aimed to evaluate the variations in soil microbial respiration after the application of dynamized high dilutions.

Materials and methods

Location, high dilutions, and experimental plan

The study was conducted in Fraiburgo/SC, Brazil (27° 01’ 36” south latitude and 50° 55’ 19” west longitude) at the Biology and Soils Laboratory of the 25 de Mayo Elementary School. Non-anthropized soil was collected to ensure diversity of microorganisms, obtaining about 65 kg of the surface layer at a maximum depth of 15 cm. Then, stones and roots were removed and the soil was homogenized and sifted at 10 mm.

Through 5 mm sieves, granulometric homogeneity was achieved in the soil samples, which were separated into 4 vats of 15 kg each. The dynamized high dilutions were obtained in the Laboratory of Homeopathy and Plant Health of Lages/Epagri. The treatments were: distilled water (T1), 30% ethyl alcohol (T2), *Calcarea carbonica* 30CH (T3), and *Silicea terra* 30CH (T4), using the same distilled water for all. The preparation followed the methods of the Brazilian Homeopathic Pharmacopoeia (Brazil, 2011).

A Tramontina® 2 L manual sprayer was used to apply 500 ml of the treatment to the soil in the vats. Of these, 5 ml corresponded to the treatment, and the rest was distilled water, thus estimating the content by field capacity in five samples. The application was made before separating the plots, which is described below. During the fumigation, the soil was turned over to ensure homogeneity in the treatments.

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After treatment, 1 kg of soil was weighed per pot, using 15 pots per treatment. Each container was labeled 1 to 4 for treatments and 1 to 15, where containers 1 to 10 were used for respiration tests and 11 to 15 as backups. The experimental design was randomized blocks with 15 replications, with one pot per plot. The order of the pots was defined by lottery, and each pot allowed two samplings to determine the respiration rates during the first phase, which was carried out from 9 to 04 of 2020 and from 17 to 4 of 2020.

After the treatments, the pots were weighed so that the weight would serve as a reference for the second (28-04-2020 to 06-05-2020) and third (16-05-2020 to 24-05-2020) applications of the treatments. Twenty grams of wet soil were weighed in the field in two centrifuge tubes per soil sample according to Schinner *et al*. (2012).

Each respiration measurement was assessed independently; therefore, 20 respiration measurements were taken per treatment. The samples were incubated for 24 h and determinations were initiated at room temperature. The experiment was treated twice more with the dynamized high dilutions, distilled water (T1), 30% ethyl alcohol (T2), *Calcarea carbonica* 30CH (T3), and *Silicea terra* 30CH (T4), where the same experimental design was preserved and the soil was not removed again. This was to simulate an agricultural environment, where the soil was turned over in the first intervention, and then only the treatment.

In the second and third applications of the dynamized high dilutions, no changes were made in the treatments or in the order of the pots. New samples were taken to determine respiration. Moisture evaporated since the first application was considered, calculated as the current weight minus the weight after the first application. A value of 20 ml was established for the second phase, based on the average evaporation of 10 pots, and it was decided to maintain the same dosage in the following applications.

Of the 20 ml of each treatment, 18 ml was distilled water and 2 ml was the treatments (the concentration was different so that it did not fall short of the 5 ml of the first treatment). Twenty milliliters of the treatments were dripped onto the surface of the pots and after 30 min, a new collection of respiration material was performed. The wait for the applied content to penetrate the soil was estimated.

Determination of soil microbial respiration

Microbial soil respiration by titration followed the principle established by Schinner *et al*. (2012). Soil samples were incubated in a closed container, where the CO_2 produced was absorbed into sodium hydroxide and quantified by titration with phenolphthalein (Jäggi, 1976).

The materials and equipment required were reagent bottle (250 ml) with screw cap and pouring ring. Centrifuge tubes or test tubes (polypropylene, outer diameter 29 mm, length 105 mm), where small holes were drilled in the tubes to allow gas exchange. Instead of tubes, fine-mesh nylon bags can also be inserted into the bottles.

Preliminary tests were conducted with nylon bags, the results of which were similar, but sample handling was poor. Therefore, all the methodology reported was carried out with centrifuge tubes. In the chemical aspect, the following reagents were used: sodium hydroxide solution (0.05 M), diluted hydrochloric acid (0.1 M), and barium chloride solution (0.5 M), prepared by dissolving 10.4 g of $BaCl_2$ in distilled water and adjusting the volume to 100 mL in a volumetric flask, a procedure performed in the chemical analysis laboratory of the Epagri/Lages Experimental Station.

Phenolphthalein indicator solution (Bayer, 1871) for CO_2 determination, 0.1 g of phenolphthalein was dissolved in ethanol (60% v/v) and the volume was increased to 100 ml with ethanol in a volumetric flask. Using a dispenser, 20 ml of sodium hydroxide solution was placed into the laboratory bottles and the tubes were inserted into the bottles. After 24 h of incubation, titration was performed, a process that was carried out for nine days.

The tubes were removed and 2 ml of barium chloride solution was added to precipitate the absorbed CO_2 as barium carbonate. Shortly thereafter, 3-4 drops of the indicator solution were added, and the remaining sodium hydroxide was titrated with diluted HCl. In titration, care was taken to remove two soilless samples containing only the aqueous solution of 0.05 M NaOH to calibrate the titration.

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The calculation was made through the equation

$$\frac{(C\text{-}S)(2.2)100}{SW\ \%dm} = mgCO_2g^{-1}dm\ 24\ h^{-1}$$

Where: C= average volume of HCl consumed by controls (ml); S= average volume of HCl consumed by samples (ml); 2.2= conversion factor (1 ml of 0.1 M HCl corresponds to 2.2 mg of CO_2); SW= initial soil weight $(g)^{-1}$ dm - soil dry matter factor.

The data were analyzed with the R Core Team software (2020) using Anova for repeated measures. Mauchly's sphericity test was applied and when it was violated, corrections were made with Greenhouse-Geisser. When the F-test showed statistical significance, Bonferroni's test was used for multiple comparisons, reducing type I error (Girardi *et al*., 2009).

Mauchly sphericity test was performed to test the property of compound symmetry, which implies the condition that the random variable is equally correlated and has equal variances considering the three analyses. The statistical significance threshold was set at 5% ($p< 0.05$).

Results and discussion

Mauchly sphericity test was applied to the three tests, and the results showed that the sphericity condition was violated, invalidating the hypothesis of normality with independent variables and constant variances. This made repeated measures analysis of variance inappropriate, so the Greenhouse-Geisser method was used. Comparisons between days and treatments were significant ($p< 0.001$), as was the effect of treatments on samples ($p< 0.001$). Splits were performed for a later Anova when $p> 0.05$.

According to Schinner *et al*. (2012), microorganisms are very sensitive. On the first day of evaluation of respiration rates, there were differences among all treatments. Treatment with distilled water showed the lowest rate, with 16.42 mg CO_2 g^{-1} dm 24 h^{-1}, whereas 30% ethyl alcohol presented 40.24 mg CO_2 g^{-1} dm 24 h^{-1}. Treatments with dynamized high dilutions recorded the highest values: 50.29 mg CO_2 g^{-1} dm 24 h^{-1} for *Calcarea carbonica* 30CH and 55.09 mg CO_2 g^{-1} dm 24 h^{-1} for *Silicea terra* 30CH (Table 1). This reaction, known as aggravation, is common in organisms treated with dynamized high dilutions (Vithoulkas, 2017).

Table 1. Soil microbial activity expressed as respiration rate (mg CO_2 g^{-1} dm 24 h^{-1}). Period from 09-04-2020 to 17-04-2020. Fraiburgo, SC (2021).

Period (day)	Treatments and respiration rates (mg CO_2 g^{-1} dm 24 h^{-1})															
	Distilled water				30% alcohol			*Calcarea carbonica* 30CH				*Silicea terra* 30CH				
1° day	16.42	±	1.24	d	40.24	±	13.89	c	50.29	±	8.93	b	55.09	±	2.09	a
2° day	4.68	±	1.57	ab	3.12	±	1.08	bc	3.86	±	1.18	abc	4.06	±	1.57	abc
3° day	3.36	±	1.52	a	1.8	±	0.82	b	2.48	±	0.56	b	2.39	±	0.46	b
4° day	3.87	±	1.92	b	6.95	±	1.78	a	6.95	±	1.85	a	4.9	±	1.19	b
5° day	1.86	±	0.93	b	2.99	±	1.33	a	3.44	±	1.11	a	2.01	±	0.78	b
6° day	4.05	±	2.65	b	8.53	±	2.72	a	8.65	±	2.58	a	5.4	±	1.91	b
7° day	4.28	±	2.56	ns	3.24	±	0.91	ns	3.44	±	1.72	ns	3.11	±	1.67	ns
8° day	3.74	±	1.66	ns	3.4	±	0.96	ns	4.88	±	2.9	ns	4.2	±	2.06	ns
9° day	3.18	±	1.73	b	3.22	±	1.19	b	5.01	±	1.81	a	4.76	±	2.19	a

Means ± standard error followed by the same letter vertically do not differ from each other according to Bonferroni's test ($p> 0.05$); CH= centesimal Hahnemannian dilution order.

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From the second to the sixth day, there was a decrease in the dose differences between treatments, and on the seventh and eighth days, there was no difference between the high dilutions and the controls. The ninth day showed significant differences again, where the dynamized high dilutions had values higher than the controls of distilled water (3.18 mg CO_2 g^{-1} dm 24 h^{-1}) and 30% ethyl alcohol (3.22 mg CO_2 g^{-1} dm 24 h^{-1}), *Calcarea carbonica* 30CH presented values of 5.01 mg CO_2 g^{-1} dm 24 h^{-1} and *Silicea terra* 4.76 mg CO_2 g^{-1} dm 24 h^{-1}; this indicates changes that persisted over time (Table 1).

On the first day of the second phase assessments, there was a significant increase in respiratory rate. There were no significant differences between the high dilutions tested, *Calcarea carbonica* 30CH and *Silicea terra* 30CH, the values were 76.94 mg CO_2 g^{-1} dm 24 h^{-1} and 75.21 mg CO_2 g^{-1} dm 24 h^{-1}, being superior to the control treatments, alcohol presented 66 mg CO_2 g^{-1} dm 24 h^{-1} and distilled water 34.4 mg CO_2 g^{-1} dm 24 h^{-1}, again the 24 h period after treatments was important for differences (Table 2).

Table 2. Soil microbial activity expressed as respiration rate (mg CO_2 g^{-1} dm 24 h^{-1}). Period from 28-04-2020 to 06-05-2020. Fraiburgo, SC (2021).

Period (day)	Treatments and respiration rates (mg CO_2 g^{-1} dm 24 h^{-1})															
	Distilled water				30% alcohol				*Calcarea carbonica* 30CH				*Silicea terra* 30CH			
10° day	34.4	±	2.71	c	66.01	±	3.49	b	76.94	±	1.62	a	75.21	±	2.28	a
11° day	13.05	±	1.3	d	14.11	±	1.27	c	15	±	0.54	b	16.29	±	0.67	a
12° day	4.34	±	0.61	ns	3.66	±	0.33	ns	4.27	±	1.03	ns	3.79	±	1.24	ns
13° day	6.63	±	0.77	c	9.41	±	1.09	a	7.82	±	0.78	b	7.14	±	0.61	bc
14° day	8.3	±	1.12	b	7.87	±	1.09	bc	10.8	±	2.07	a	6.96	±	0.75	c
15° day	7.21	±	0.87	b	8.48	±	0.81	a	8.8	±	0.8	a	7.49	±	0.58	b
16° day	5.41	±	0.54	b	5.57	±	0.39	b	5.8	±	0.66	b	6.92	±	1.08	a
17° day	5.7	±	0.38	ns	5.77	±	0.34	ns	5.9	±	0.67	ns	6.22	±	0.92	ns
18° day	7.91	±	1.97	cd	9.98	±	3.18	bcd	10.86	±	1.41	abc	12.37	±	3.15	ab

Means ± standard error followed by the same letter vertically do not differ from each other according to Bonferroni's test (p> 0.05); CH= centesimal Hahnemannian dilution order.

The action of alcohol increased basal respiration, but dynamized high dilutions achieved an increase of 16.5% in 24 h compared to 30% alcohol and the incubation time required for constant basal respiration depended on the easily degradable carbon content in the soil.

If soils were stored for a few days at room temperature before analysis, basal respiration will be linear after 10 to 15 h and in some cases, 1 to 2 days (Schinner *et al*., 2012). This may explain why rates stabilize after the first two days. On the second day, there were differences between all treatments; however, the values were lower than those of the first day of the second phase, indicating that the most effective action on respiration rates occurs in the first 24 h after the treatments.

On the fourth day, treatment with 30% ethyl alcohol stood out with 9.41 mg CO_2 g^{-1} dm 24 h^{-1}. On the seventh day, *Silicea terra* 30CH again showed the highest respiration rate. In general, the best variations were observed in the first two or three days (Table 2). On the first day of the third phase, a significant increase in respiratory rates was observed, similar to the previous phases (Tables 2 and 3), with the first observations standing out for their significantly different values in the three applications of dynamized high dilutions.

Revista Mexicana de Ciencias Agrícolas

DOI: https://doi.org/10.29312/remexca.v16i3.3641

Table 3. Soil microbial activity expressed as respiration rate (mg CO_2 g^{-1} dm 24 h^{-1}). Period from 16-05-2020 to 24-05-2020. Fraiburgo, SC (2021).

Period (day)	Treatments and respiration rates (mg CO_2 g^{-1} dm 24 h^{-1})															
	Distilled water				30% alcohol				*Calcarea carbonica* 30CH				*Silicea terra* 30CH			
19° day	29.49	±	1.84	c	54.27	±	6.67	b	64.47	±	4.86	a	65.52	±	1.37	a
20° day	14.67	±	0.88	b	14.56	±	0.58	b	15.56	±	0.83	a	16.03	±	0.51	a
21° day	12.03	±	1.11	a	10.48	±	0.77	b	11.73	±	0.5	a	11.41	±	0.31	a
22° day	12.53	±	1.28	ns	13.31	±	1.92	ns	13.42	±	2.05	ns	12.55	±	1.55	ns
23° day	11.2	±	0.79	ns	11.6	±	1.26	ns	11.49	±	0.92	ns	11.29	±	0.58	ns
24° day	10.62	±	3.69	bc	12.85	±	4.26	abc	14.33	±	4.26	ab	12.49	±	2.67	abc
25° day	9.89	±	2.5	bc	11.52	±	1.13	abc	12.18	±	1.11	ab	10.45	±	3.08	abc
26° day	7.17	±	0.69	b	7.7	±	0.72	b	6.99	±	2.04	b	8.5	±	1.11	a
27° day	6.2	±	1.37	ns	7.02	±	1.96	ns	6.24	±	2	ns	7.28	±	2.58	ns

Means ± standard error followed by the same letter vertically do not differ from each other according to Bonferroni's test (p> 0.05); CH= centesimal Hahnemannian dilution order.

In the period from 16-05-2020 to 24-05-2020, the first and second days can be highlighted, in which the *Calcarea carbonica* 30CH and *Silicea terra* 30CH treatments presented significantly higher respiration rates compared to the controls. On the first day of the third phase, *Calcarea carbonica* 30CH 64.47 mg CO_2 g^{-1} dm 24 h^{-1}, *Silicea terra* 30CH 65.52 mg CO_2 g^{-1} dm 24 h^{-1}, 30% ethyl alcohol 54.27 mg CO_2 g^{-1} dm 24 h^{-1}, and distilled water 29.49 mg CO_2 g^{-1} dm 24 h^{-1}. It can be said that the treatment composed of only 30% ethyl alcohol showed a significant difference when compared to distilled water alone.

In other words, part of the effect attributed to respiration rates is due to the alcohol present in the treatment. However, even so, high dilutions present different values in the first two days of treatment, the result itself is proof that the dosage for use in agriculture should be less than 30% alcohol. It is important to note that 30% of alcohol refers to the preparation, the dilution applied was 1% and then 10% (10% not to increase moisture, 1% is recommended); in the present study we chose to use this percentage due to the longevity given to the preparation (Brazil, 2014).

On the eighth day, *Silicea terra* 30CH showed a significantly higher value than the other treatments, 8.5 mg CO_2 g^{-1} dm 24 h^{-1} (Table 3). In this study, a sudden increase in respiration rate was observed on the first day after applying dynamized high dilutions in the soil compared to controls. This can infer immediate reactivity in response to treatments, similar to the phenomenon of reactivity in humans and animals, identified as a side effect. The side effect occurs in response to the action of drugs on the body (primary effect).

This occurs in organisms sensitive to homeopathic preparations, suggesting that soil microorganisms were affected by the preparations, showing temporary effects on soil respiration, and evidencing a healing and reorganizing potential of microbial flora (Boff, 2009). The idea of aggravation in homeopathy implies alterations in vital signs after ingesting the drug, a phenomenon that does not seem to be exclusive to mammals and other animals. The results of the first day of evaluation, shown in Tables 1, 2 and 3, indicate that these phenomena can also manifest themselves in microorganisms.

Aggravation is studied little in works that seek to criticize the effects derived from the use of homeopathy (Vithoulkas, 2017). During the determination of basal respiration in the laboratory, an increase in CO_2 production was observed during the first hours. This is due to an increase in nutrient availability after mixing and a rapid adjustment of the CO_2 balance (Schinner *et al*., 2012). This occurred in the three phases of the experiment carried out from 09-04-2020 to 17-04-2020, 28-04-2020 to 06-05-2020, and 16-05-2020 to 24-05-2020, respectively.

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The relationship between microbial activity and humidity in the environment is relevant and has been demonstrated in several studies (Peña *et al*., 2005). Higher respiration rates were expected in the first assessments. In forest soils with a high litter fraction, this linearity is not achieved even after long incubation periods. In less biologically active arable soils, levels with high sensitivity are suitable for measuring linear respiration (Schinner *et al*., 2012). In our experiment, the origin of the soil showed that there was no linearity over the days.

Conclusions

The *Silicea terra* 30CH and *Calcarea carbonica* 30CH treatments showed a different behavior compared to the controls of distilled water and 30% ethyl alcohol. It was crucial to repeat the experiments to evaluate the effect of high dilutions, especially in the first 24 h, where the results were more expressive and statistically different from the controls, with distilled water generating the lowest respiration rate. Although alcohol may have influenced some results, the respiration peaks suggest a significant effect of dilutions. Throughout the three phases, differences in CO_2 rates were observed during the nine days, with a markedly smaller effect after this period, so it is recommended to limit incubation to two or three days in future experiments.

Acknowledgements

FAPESC through the Rede Guarani Serra Geral project / TO 2015TR1067, CNPq (procedure n. 304018/2015; 307376/2017-6) and the UNIEDU Postgraduate Program for the first author scholarship.

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DOI: https://doi.org/10.29312/remexca.v16i3.3641

Assessment of high dilutions on soil microbial respiration

Journal Information
Journal ID (publisher-id): remexca
Title: Revista mexicana de ciencias agrícolas
Abbreviated Title: Rev. Mex. Cienc. Agríc
ISSN (print): 2007-0934
Publisher: Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias

Article/Issue Information
Date received: 01 January 2025
Date accepted: 01 April 2025
Publication date: 23 April 2025
Publication date: Apr-May 2025
Volume: 16
Issue: 3
Electronic Location Identifier: e3641
DOI: 10.29312/remexca.v16i3.3641

Categories

Subject: Articles

Keywords:

Keywords:

dynamized dilutions
microbial respiration
soil health.

Counts

Figures: 0
Tables: 3
Equations: 1
References: 23
Pages: 0