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CIÊNCIAS AGRÁRIAS

Interference of experimental designs on plant elicitation studies performed with wheat

Interferência de desenhos experimentais nos resultados de estudos de indução de resistência conduzidos com trigo

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ABSTRACT

The use of plants' natural defenses, including the utilization of different compounds and stimuli, has increased significantly since the discovery of the metabolic pathways involved in the synthesis of specialized compounds by plants. When elicited, plants release a large amount of volatile organic compounds (VOCs) in the air, which can act as great communicators and elicitors to neighboring plants and organisms. These VOCs can cause significant interference when testing new compounds against biotic and abiotic agricultural stressors. To mitigate these interferences and ensure that the observed effects and results are solely attributed to the treatments being evaluated, it is crucial to perform plant elicitation trials in total controlled conditions with the use of physical barriers to prevent VOCs exchange between treatments. To investigate the potential interference caused by VOCs, a study was conducted comparing two experimental sets: one performed in an open field using a randomized complete block design (RCBD), and another conducted in total controlled conditions using a completely randomized design (CRD) with adaptations. The study found that, on average, an interference of over 22% was observed for the assessed parameters. This suggests that the exchange of VOCs between

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treatments in the open field trial leads to significant interference and affects the reliability of the results.

Keywords: bioestimulant; induction of resistance; plant-plant communication; VOC interference; *Solieria chordalis*.

RESUMO

O uso das defesas naturais das plantas, com induções a partir da utilização de diferentes compostos e estímulos, aumentou significativamente desde a descoberta das vias metabólicas envolvidas na síntese de compostos especializados pelas plantas. Quando induzidas, as plantas liberam compostos orgânicos voláteis (VOCs) na atmosfera que podem atuar como comunicadores e eliciadores de plantas e organismos vizinhos. Esses VOCs podem causar interferências significativas nos resultados obtidos. Para mitigar essas interferências e garantir que os efeitos e resultados observados sejam atribuídos exclusivamente aos tratamentos avaliados, é fundamental realizar ensaios de eliciação vegetal em condições controladas com o uso de barreiras para evitar a troca de VOCs entre tratamentos. Para investigar a possível interferência causada por VOCs foi realizado um estudo comparando dois experimentos: um realizado em campo aberto (delineamento de blocos casualizados – DBC) e outro conduzido em condições controladas (delineamento inteiramente casualizado – DIC) com adaptações. O estudo constatou que, em média, foi observada uma interferência de mais de 22% nos parâmetros avaliados. Isso sugere que a presença de VOCs no ambiente de campo causa uma interferência significativa e afeta a confiabilidade dos resultados.

Palavras-chave*: bioestimulante; indução de resistência; comunicação planta-planta; Interferência por VOCs; Solieria chordalis.*

1. INTRODUCTION

Plant elicitation was firstly described in the beginning of the 20th century with the use of *Botrytis cinerea* attenuated spores to increase plant resistance to this species of fungi (Conrath, 2006). Nevertheless, it was only in the 1970’s that plant elicitation studies really took off with the discovery of the pathways involved in the production of specialized metabolites by plants, previously considered to be metabolic wastes (Ducatti, 2023). To date, more than 200 thousand compounds synthesized by plants through their specialized metabolism have been identified (Hartmann, 2007). Some of these compounds are classified as volatile, and once synthetized are quickly released by plants in the form of gas (Markovic *et al*., 2019).

Once elicited, plants are led to increase their primary and specialized metabolisms, which leads to an increased exchange of gases with the atmosphere. Studies have demonstrated that plants release back into the atmosphere in the form of volatile molecules approximately 20% of the CO_2 daily absorbed (Baldwin, 2010). These compounds, also known as Volatile Organic Compounds (VOCs), are highly effective airborne communicators (Erb; Kliebenstein, 2020). Holopainen and Blande (2012) showed that VOCs can persist in the air for extended periods, travel considerable distances, and lead to substantial interference with neighboring plants, therefore, affecting experimental outcomes (Markovic *et al*., 2019; Vicherová *et al*., 2020; Jin *et al*., 2021; Brambilla *et al*., 2022).

Volatile Organic Compounds are associated to many advantages to plants and the environment, which include: 1) facilitate communication among neighboring plants and other organisms, such as insects; 2) prime plants for biotic and abiotic stresses; 3) attract or repel predators; 4) attract pollinators; 5) stimulate the primary metabolism; and 6) react to and form different compounds in the atmosphere (Razo-Belman; Ozuna, 2023).

Although the mechanisms through which plants recognize VOCs are still not yet completely understood (Loreto; D'Auria, 2022), it is well known that VOCs can significantly impact plant elicitation studies (Ducatti, 2023) and mask the results obtained during these trials. Therefore, when testing new molecules, it is essential to ensure that the results generated are not influenced by interferences that may lead to the rejection of the tested molecule as a potential candidate against diseases, pests, or biotic/abiotic stresses, or for enhancing plant development.

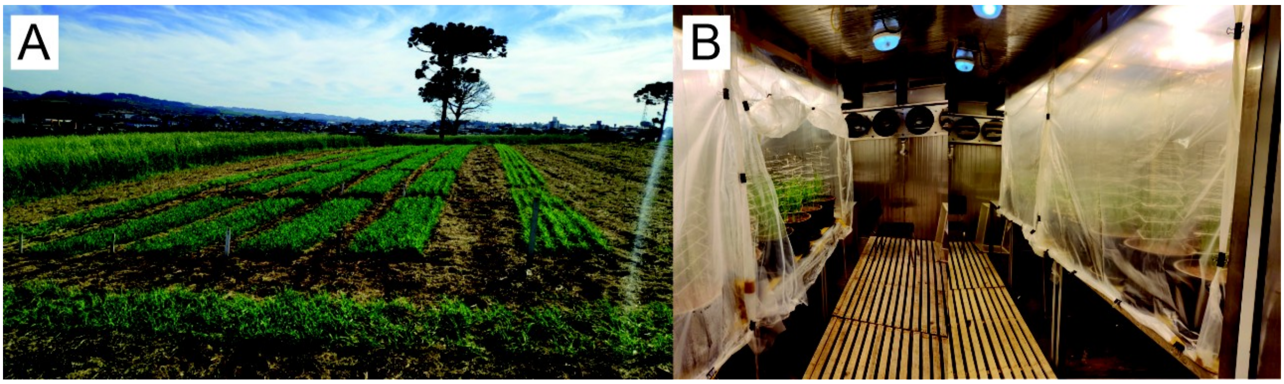
With this in mind, this study was arranged to directly assess the interference caused in the results obtained for wheat cultivation due to the presence and VOCs exchange between treatments. To achieve this, two experimental settings were used, one developed under a randomized complete block design (RCBD – allowing VOCs exchange), and another under a completely randomized design (CRD – blocking VOCs exchange), with adaptations.

2. MATERIAL AND METHODS

2.1. EXPERIMENTAL DESIGNS AND TREATMENTS

Different experiments and experimental designs were used to evaluate the potential interferences arising from VOCs exchange in plant elicitation studies. The first experiment followed a randomized complete block design (RCBD) with four treatments and four replicates using 5.0 x 1.0 m plots in open field. The second experiment was developed as a completely randomized design (CRD) with four treatments and twelve replicates consisting of twelve 20-liter pots per treatment, each pot containing twelve plants. This second experiment was conducted in a phytotron with specific adaptations to prevent VOCs exchange from treatments (Figure 1).

Figure 1 – Demonstration of the experimental designs used during the experiments. Randomized complete block design (A) and Completely randomized design with adaptations (B).



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The phytotron adaptations involved constructing small growth chambers (2.5 x 1.0 m) using a 150-micron greenhouse plastic film. These chambers were designed to house each treatment separately, preventing intercommunication between treatments through VOCs. All plants were kept in these chambers since seeds were sown. In the open-field experiment, intercommunication between treatments was allowed.

The treatments for both experiments were as follows:

- Treatment A: Untreated control.
- Treatment B: Three applications of the fungicide (Trifloxystrobine 150 g L^{-1} and Prothioconazole 175 g L^{-1}) – 0.5 L ha^{-1} at the beginning of the tillering (GS20), booting (GS40), and flowering stages (GS60) (Zadoks *et al.*, 1974).
- Treatment C: Single application of the elicitor – Red Seaweed Biostimulant (RSB) (Algomel PUSH® - *Solieria chordalis*) – 1.0 L ha^{-1} at the beginning of the tillering stage (GS20).
- Treatment D: Combination of Treatment B and Treatment C.

Fungicide and seaweed applications were performed using a portable CO_2 sprayer calibrated to deliver a solution of 200 L ha^{-1}. In the phytotron, all treatments were subjected to the same growing conditions, including Photosynthetic Active Radiation at 700 µmol s^{-1} m^{-2}; Temperature at 32 ± 4 °C during light exposure and 17 ± 2 °C during dark exposure; Photoperiod of 12 hours of light and 12 hours of darkness; Air moisture of 85 ± 5%; Soil moisture maintained at field capacity. The cultivars used for the experiments were TBio Audaz® (phytotron) and TBio Noble® (open field). The reason why these experiments used different wheat cultivars lies on the fact that TBio Noble® seeds were not available when the experiment was set in the phytotron.

For the phytotron experiment, each pot was filled with a medium composed of a 9:1 mixture of soil (from the open field experimental area) and a commercial organic substrate. All pots received a nutrient supplementation (0.1 L) composed of 1.5% w/w KCl, 1.5% w/w $(NH_4)_2SO_4$, 0.05% w/w H_3BO_3, and 0.05% w/w Na_2MoO_4 after 25, 41, and 56 days of wheat emergence. The open field experiment received 62.0 kg ha^{-1} of urea, 100.0 kg ha^{-1} of N-P_2O_5-K_2O (09-33-12), and 16.0 kg ha^{-1} of KCl at sowing. Additionally, an extra application of 100.0 kg ha^{-1} of urea was performed 30 and 40 days after emergence.

2.2. ENZYME ACTIVITY AND SALICYLIC ACID CONTENT

The activity of superoxide dismutase (SOD), catalase (CAT), and phenylalanine ammonia-lyase (PAL) were assessed. Superoxide dismutase and catalase were measured following the protocols described by Bettini *et al.* (2014). The activity of phenylalanine ammonia-lyase was quantified through the protocol stablished by Rodrigues *et al.* (2006). Salicylic acid (SA) quantification was conducted using the methodology proposed by Warrier *et al.* (2013). A total of 234 quantifications were performed for each enzyme, and 186 for SA content.

2.3. PHYSIOLOGICAL PARAMETERS AND POWDERY MILDEW SEVERITY

Internal carbon (Ci), transpiration (E), stomatic conductance (Gs), and net photosynthetic rates (A) were measured at different time intervals using an Infra-red Gas Analyzer adjusted to a light saturation of 1500 µmol m^{-2} s^{-1} (Lcpro-SD Serial n° 33961 – ADC BioScientific Ltd., Hoddesdon, UK). The SPAD index was assessed in different periods during crop development using a chlorophyll meter (atLEAF PLUS – Wilmington, Delaware, USA). A total of 190 measurements were performed for Ci, E, Gs and A, and 372 for SPAD index.

Powdery mildew severity assessments were conducted with the scale proposed by Domiciano *et al.* (2013). A total of 150 assessments were performed during crop development. For the experiment conducted in the phytotron, powdery mildew spores were brought by the soil used to produce the substrate used to fill in the pots. Spores were homogenized across all pots by fully mixing the commercial organic substrate and the soil from the experimental area in a 600-L concrete mixer for over 10 minutes.

2.4. STATISTICAL ANALYSIS

Different comparations were performed for the variables analyzed under different experimental conditions. The first consisted of a basic comparison between the different treatments. For this comparison, the untreated control mean was considered as 100%, and the increase or decrease percentage of each variable was calculated in relation to this reference. This analysis aimed to show the differences (amplitude) between the same variables when experiments were performed under total controlled conditions (CRD) or in an open field (RCBD).

The second aimed to conduct a statistical comparison between the experimental designs. To perform this, each measurement for each variable was subtracted from the mean of the untreated control for that respective variable. The following formula was used:

$$Y = A_i - \bar{x}_{uc}$$

where, Y represents the subtraction value, A_i denotes each measurement performed for each variable during the experiment, and $\bar{x}_{uc}$ represents the mean of the untreated control for each variable.

The statistical analysis for the second comparison involved the elimination of outliers followed by normality tests (Shapiro-Wilk) to ascertain normal distribution of the data. If the data did not exhibit a normal distribution, appropriate transformations were applied. Data that did not conform to a normal distribution, even after transformation, were analyzed using non-parametric statistical tests (Kruskal-Wallis). Data that exhibited a normal distribution were analyzed using analysis of variance followed by the F-test. All statistical analyses were performed using R-software.

3. RESULTS AND DISCUSSION

The results indicate that certain variables were significantly impacted by the experimental design employed in the trials. Specifically, conducting elicitation experiments in open field conditions without the use of physical barriers to block gas exchange (VOCs) between treatments had a substantial influence on the obtained results. For instance, powdery mildew severity was affected by more than 80% when trials were conducted in open fields (Table 1).

Table 1 – Mean differences (%) observed in relation to the untreated control for the variables analyzed in trials conducted with different experimental designs (RCBD vs. CRD).

Design used*	Treatment	CAT	PAL	SOD	SA	Ci	E	Gs	A	P. Mildew	SPAD
		Difference (%) between the mean of each treatment in relation to the untreated control									
RCBD	Fungicide (3x)	-10,5	6,40	-9,90	9,90	-1,91	6,48	4,02	6,39	-22,8	2,92
	Elicitor (RSB) (1x)	-2,90	5,40	0,21	-	-1,12	3,95	-2,30	2,12	4,35	0,77
	Fung. + Elicitor	-9,10	-5,58	-5,14	-5,63	-1,23	-1,67	-0,23	2,00	-30,4	2,73
CRD	Fungicide (3x)	34,9	-0,34	-21,9	7,69	12,1	-0,44	0,62	-6,63	-100	-1,26
	Elicitor (RSB) (1x)	28,3	4,45	-40,6	20,5	20,8	13,5	20,0	18,5	-100	-2,01
	Fung. + Elicitor	17,3	-5,78	-46,9	16,1	28,1	0,54	3,90	-11,6	-100	-4,31
		Difference (%) between the mean of each treatment (RCBD vs. CRD)									
	Fungicide (3x)	45,4	6,75	31,8	2,21	14,1	6,93	3,39	13,0	77,1	4,18
	Elicitor (RSB) (1x)	31,3	0,94	40,8	-	21,9	9,63	22,3	16,3	104,3	1,24
	Fung. + Elicitor	26,4	0,19	52,1	21,7	29,3	1,13	4,13	13,6	69,5	7,05
		Total mean difference (%) observed between all treatments (RCBD vs. CRD)									
		34,4	2,63	41,6	11,9	21,7	5,89	9,97	14,3	83,6	4,16

* RCBD: randomized complete block design; CRD: complete randomized design.
CAT (catalase), PAL (phenylalanine ammonia-lyase), SOD (superoxide dismutase),
SA (salicylic acid), Ci (internal carbon concentration), E (transpiration),
Gs (stomatic conductance), A (net photosynthetic rate),
P. Mildew (powdery mildew incidence), SPAD (Soil Plant Analysis Development).

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The influence of the different experimental designs had a significant impact on several variables analyzed during the experiments. It is worth noting that previous studies have already highlighted the potential interference that volatile organic compounds (VOCs) cause to the outcomes of plant elicitation studies (Ducatti, 2023). For instance, Piesik *et al.* (2013) showed that after wheat and barley inoculation with *Fusarium* spp. spores, uninfected plants located at distances of one and three meters from the inoculated plants had their VOCs production increased by 25-50% and 10-25%, respectively. Markovic *et al*. (2019) influenced the production of VOCs in maize plants by using a soft brush to simply touch plants. After this elicitation by touch, VOCs receiving neighboring maize plants had their specialized metabolism increased. In 2022, Brambilla *et al*. used powdery mildew spores (*Blumeria graminis* f. sp. hordei) to infect barley plants, increase their production of VOCs, and raise the resistance against this same species of fungi in VOCs receiving neighboring barley plants.

These interferences caused by VOCs potentially lead to the rejection of promising molecules/products as possible solutions for agricultural issues, such as the incidence of the commonly found *B. graminis* fungi in wheat and barley fields. These interferences also lead to making agriculture less sustainable (Shukla *et al.*, 2021; Ducatti *et al.*, 2024). When rejecting an organic and natural occurring molecule, or any other source of sustainable product, as a potential defense against a pathogen/insect only because it was tested in a non-appropriated form, one is inducing farmers to keep using synthetic pesticides as the “only” possible solution to their problem. Therefore, it is crucial to consider and mitigate the effects of VOCs when evaluating the efficacy of new substances in addressing agricultural problems.

When conducting plant elicitation studies in "not-controlled conditions" or without a complete isolation of treatments, researchers allow the intercommunication between plants and treatments. This can result in the generation of biased results that can mask the true potential of tested products and molecules. It is important to acknowledge that the presence of a blend of volatile organic compounds (VOCs) might have more pronounced elicitation effects compared to the application of isolated compounds to plants (Brosset; Blande, 2022).

After observing significant differences in certain variables when experiments were conducted using different experimental designs, an additional comparison was conducted to determine the statistical significance of these differences. This comparison is presented in Table 2.

Significant differences were observed for many variables when comparing the different experimental designs. These findings emphasize the critical importance of selecting an appropriate experimental design and adapting them when conducting elicitation studies. Specifically, it is crucial to consider the implications of VOCs and their potential for intercommunication and interference between treatments.

To obtain more accurate and reliable results regarding the true potential of a product or molecule, it is recommended to implement measures that completely block VOCs exchange between treatments. By doing so, researchers can minimize the effects of VOCs and ensure that the observed effects are attributed solely to the treatments being evaluated. This approach allows for more precise assessments of the efficacy and potential of tested products in addressing agricultural challenges.

It does not mean that plant elicitation experiments can't be performed in open fields using designs such as Randomized Complete Block Design (RCBD), Latin Square, or any other. The crucial aspect here is to block gas exchange between treatments. If a plant elicitation experiment is conducted using a Completely Randomized Design (CRD) without blocking gas exchange, one may end up generating biased results as well. However, total controlled conditions, such as a phytotron or a controlled environment chamber, offer greater ease to adapt the experiment to effectively block gas exchange and maintain precise control over experimental conditions.

Table 2 – Statistical comparison of the variables analyzed in trials performed with different experimental designs (RCBD vs. CRD).

Variable	Factor	Df	Sum Sq	Mean Sq	F-value	*P or ChiSq* value
Ci[2]	-	1	-	-	-	0,0006**
E[1]	Year	1	0,10	0,10	0,101	0,7500
	Error	188	189,89	1,01		
	Total	189	190,00			
Gs[1]	Year	1	0,54	0,54	0,537	0,4643
	Error	188	189,45	1,00		
	Total	189	190,00			
A[1]	Year	1	0,71	0,71	0,707	0,4015
	Error	188	189,28	1,00		
	Total	189	190,00			
SPAD index[1]	Year	1	10,25	10,25	10,485	0,0013*
	Error	370	361,74	0,97		
	Total	371	372,00			
CAT[1]	Year	1	26,73	26,73	29,930	<0,0001*
	Error	232	207,26	0,89		
	Total	233	234,00			
SOD[2]	-	1	-	-	-	0,0033**
PAL[2]	-	1	-	-	-	<0,0001**
AS[2]	-	1	-	-	-	0,5234
P. mildew[2]	-	1	-	-	-	<0,0001**

* significative for the F-test (< 5%)
** significative for Kurskal-Walis (< 5%)

[1] these variables had to be transformed using "Johnson's transformation" to acquire a normal distribution.
[2] these variables were analyzed via non-parametric analysis.

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4. FINAL CONSIDERATIONS

It is recommended to conduct plant elicitation trials in controlled conditions where physical barriers can be used to block gas exchange between treatments. By implementing these barriers, the possibilities of intercommunication between treatments are minimized, allowing for the acquisition of unmasked and more reliable results. Plant elicitation trials performed with wheat in open field, or without fully blocking VOCs exchange between treatments, may lead to a mean interference of over 22% in the results obtained.

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