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Response of Plant Immunity Markers to Early and Late Application of Extracellular DNA from Different Sources in Tomato (*Solanum lycopersicum*)

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Abstract: As a recently explored agricultural practice, the controlled elicitation of plants offers high potential in multiple crop needs as growth promotion, activation of defenses and the production of specific metabolites. Extracellular DNA has been identified as a plant immune system elicitor but some aspects of the plant response have not been explored. In the present work, five DNA treatments were applied in tomato plants and the response of catalase, superoxide dismutase, phenylalanine ammonia lyase activities, hydrogen peroxide, total phenolics and flavonoid contents in leaves were spectrophotometrically measured. Treatments differed by the source and concentration of DNA and plant phenological stage of application. Furthermore, mathematical modeling and principal component analysis were performed to explore the behavior of each variable and their interaction. The most effective treatment was the self-DNA application in young plants based on the intensity and duration of immune system activation. The information given by the measured variables correlated positively with the phenylpropanoid pathway markers and negatively with catalase and superoxide dismutase activities. Results reported here propose an easy way to evaluate plant immune response activation by DNA and any other elicitor and provide useful information for future development of controlled elicitation strategies in crop production.

Keywords: eDNA; plant elicitors; plant immune system; plant priming; DAMPs; PAMPs

1. Introduction

The green revolution is focused on increasing worldwide food supply of agricultural systems; however, how those changes would affect the environment was not a primary consideration. Nowadays, consumer habits and production practices keep increasing and changing the population's food consumption [1]; due to this, agriculture systems require new management strategies to meet this demand, while looking for environmental, health and ecological balance through a more sustainable primary production. In this context, several technologies have been proposed to achieve these goals. One of the most promising is the use of controlled elicitation strategies in crops, which consists in an adequate management of stress factors that, in the right dose, will function as eustressors [2]. It has been seen that eustressors can activate defense mechanisms and some have also been shown to enhance plant development and growth [3–8].

Recently, the role of extracellular DNA (eDNA) as a signal molecule has accumulated experimental evidence. The mechanisms that plants have evolved to sense eDNA have showed a high specificity to the DNA origin. Different responses have been identified if the applied eDNA is conspecific; these treatments are called self eDNA. Otherwise, non-conspecific eDNA is called non-self eDNA. On the one hand, some early defense activated by self eDNA such as plasma membrane depolarization and Ca^{2+} accumulation [9], oxidative burst, activation of mitogen activated protein kinases (MAPKs) and induction of extrafloral nectar [10] and, as later responses, changes in genome methylation patterns [11] and genetic expression [12–14] have been identified. Due to the latter evidence, researchers have suggested a new role for eDNA as a damage-associated molecular pattern (DAMP) [15,16]. On the other hand, non-self eDNA has been proved to activate plant defense mechanisms when it is extracted from microbial pathogens and applied to plants [17], or to only have bacterial characteristics [18]. This behavior has been described as similar to pathogen or microbial-associated molecular patterns (PAMP/MAMP) [19]. In addition, recent studies have shown similar, less intense responses to non-self-DNA without microbial characteristics, such as DNA extracted from herring [13] and several plant species [14]. The application of random eDNA to plants has even been suggested as a potential treatment for general bio-stimulation [20].

Although recent works describe interesting features of eDNA plant perception and the corresponding defense mechanisms, the application perspectives for agriculture have not been developed in many cases. The main purpose here is to take the first steps towards the application of a natural plant response using a controlled elicitation design. For an agriculture treatment to be called controlled elicitation, fine characterization of the biological responses and data analysis considering the hormesis dose response perspective are needed. Hormesis is a biphasic dose response phenomenon characterized by low dose stimulation but also a high dose inhibition [21]. Therefore, more information on the possible responses of plants to these treatments is desirable.

In the present work, three markers of the phenylpropanoid pathway (PPP) and three markers of the Reactive Oxygen Species (ROS)/antioxidant system were measured as a response to self and non-self eDNA applications on tomato plants in two phenological stages, along with its behavior through time. The protocol for DNA extraction used in the present study was as simple as possible, focusing on the possibility to scale it up for future agricultural applications. Therefore, this research aimed to compare the effect of eDNA from different DNA sources applied to two different phenological stages in tomato plants to contribute to the design of strategies for eDNA application during cultivation based on the response of plant immunity indicators in tomato as the plant model of study.

2. Materials and Methods

2.1. Biological Material

To compare the effect of application of eDNA from different sources in plants, applied eDNA was extracted from 3 different sources detailed in Table 1. The National Institute for Forestry, Agriculture and Livestock Research (INIFAP) kindly provided the fungus strain used in the study.

Table 1. Sources of DNA for bioassays.

DNA Source	Role	Biomass Source
Tomato (*Solanum lycopersicum* var. Rio grande).	Self eDNA	From crop pruning
Pathogen (*Fusarium oxysporum*)	Non-self eDNA	Micelia grown in PDB and incubated at room temperature with agitation (30 RPM) for 3 weeks.
Mixed plants	Non-self eDNA	20% of lettuce (*Lactuca sativa*) leaves, 40% of celery (*Apium graveolens*) leaves, and 40% of cucumber (*Cucumis sativus*) leaves; from crop pruning.

2.2. Extraction and Fragmentation of DNA

DNA was extracted following the methodology used by Rodrigues et al. in 2018 [22], with some changes to adjust the protocol to a higher volume of extracted DNA. For plant DNA extraction, 50 g of biological material were collected and blended with 100 mL of Buffer SDS (200 mM Tris-HCl pH 8.5; 250 mM NaCl; 25 mM EDTA; 0.5% (*w*/*v*) SDS) in a common kitchen blender Oster 6662-13 for 10 s. For fungi DNA extraction, 10 mL of 20% SDS were added to the mix. The obtained mix was passed through a metal sieve with aperture of 1 mm. Then, 25 mL of the liquid phase was transferred to Falcon tubes and 15 mL of 3 M sodium acetate was added, gently mixed and rested for 10 min at −20 °C. The mix was centrifuged for 15 min at 16,500× *g* and 4 °C in a ScienceMED DM0412S Centrifuge. Supernatant was recovered in a clean tube and then half of the supernatant volume was added as cold isopropanol. The tubes were incubated for 1 h at −20 °C, centrifuged at 16,500× *g* for 7 min (4 °C) and new supernatant discarded, once the fully dry DNA pellet was dissolved in 20 mL of sterile distilled water.

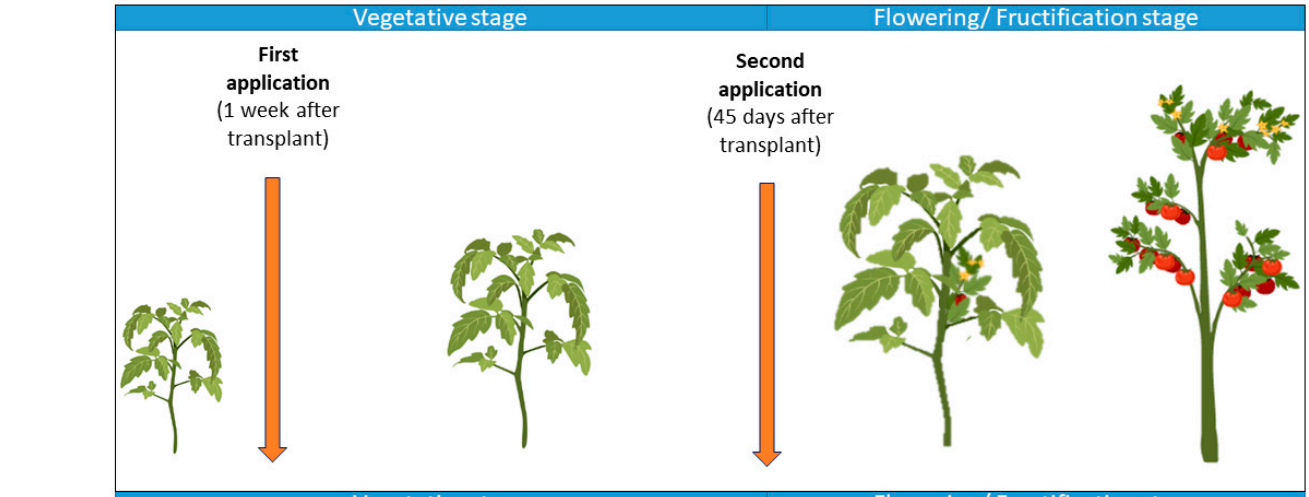
As previously confirmed [10], eDNA must be fragmented at a <1000 bp size to elicit plant responses. Thus, DNA solutions were sonicated with a Hielscher UP200Ht sonotrode with pulses of 26 KHz at 10 W at 50% amplitude every 1 s for 30 min. DNA structure and concentration were measured by spectrophotometry at 260 nm with a Thermo Scientific NanoDrop (AccesoLab, Queretaro, México) 2000c Spectrophotometer and by DNA visualization in agarose gels.

2.3. Biological Assays

Tomato seeds of the variety Río Grande (King Seeds Inc. Guadalajara, México) were germinated and, when 4–6 true leaves where visible, the plantlets were transferred to plastic bags with crushed gravel as substrate. Plants were grown under greenhouse conditions: natural light, average temperature of 24 °C in daytime and 12 °C in nighttime, and fertilized with Poly-Feed Greenhouse grade (Haifa Group Inc. Haifa, Israel) in the doses suggested by the producer.

In Figure 1, the timeline for this experiment is represented. One week after transplant, the first application of eDNA was carried out as follows: plants of each treatment were sprayed to drop point with 10 mL of eDNA solution. Control plants were sprayed with 10 mL of deionized water. Based on previous studies in our group, four different treatments were applied to tomato plants: (1) 50 ppm self eDNA, (2) 15 ppm and 150 ppm of *F. oxysporum* eDNA and, (3) 100 ppm mixed plants eDNA (as described in Table 1) [11,16,17]. A second application to drop point of the treatments was carried at 45 days after transplant; at this time, all plants had flowered and with at least 1 fruit. Additionally, a group of non-treated plants during the first application were sprayed with 50 ppm of self eDNA 45 days after transplant, generating another treatment called self eDNA late application. This latter treatment would help us to evaluate plant response at different phenological stages.

Plants were established by triplicate for each treatment and for each sampling time. The experiment was designed in a completely random array. Samples consisted of 2 leaves for each sampled plant and were collected at 1 and 5 h and 1, 5 and 10 days post-application. After the second application samples, were collected at 1hour and at 5 and 10 days.



Sampling times	1 hour post first application (1HPA)	5 hours post first application (5HPA)	1 day post first application (1DPA)	5 days post first application (5DPA)	10 days post first application (10DPA)	1 hour post second application (1HPSA)	5 days post second application (5DPSA)	10 days post second application (10DPSA)

Figure 1. Timeline graphical representation of the experiment carried out in this work. The times of eDNA application are abbreviated from now as follows: 1 h after first application (1 HPA), 1 day after first application (1 DPA), 1 h after second application (1 HPSA) and 5 days after second application (5 DPSA). Some elements were created with BioRender.com.

2.4. Plant Immunity Indicator Measurements

Hydrogen peroxide (HP) determination was made following the protocol reported by Junglee et al. 2014 [23]. Briefly, tissues were grounded with liquid nitrogen in a mortar and pestle; 150 mg of frozen powder was homogenized with 1 mL containing 0.25 mL of 0.1% Trichloroacetic, 0.5 mL of 1 M KI and 0.25 mL of potassium phosphate buffer (10 mM). The mix was vortexed and incubated for 10 min at room temperature. After this, each tube was centrifuged at 14,000× *g* for 15 min and 250 µL of supernatant were transferred to a well in a microplate and rested for 20 min; the microplate was measured at 350 nm. At the same time, controls were prepared with distilled water instead of KI for tissue coloration background.

For determination of enzyme activities, 0.3 g of pulverized vegetal tissue were vortexed with 1 mL of phosphates buffer for 1 min. The obtained mixture was separated by centrifugation and supernatants were collected as enzyme extracts (EE) for assays. Activities were determined by spectrophotometric analyses adapting classical methodologies used since Cakmak and Horst [24], Beauchamp and Fridovich [25] and Dickerson et al. [26].

The activity of catalase (CAT) was determined by monitoring the oxidation of H_2O_2 measuring absorbance at 240 nm. In a well of a 96 well microplate, the following mixture was made: 200 µL of reaction buffer, 20 µL of hydrogen peroxide and 100 µL of EE. Absorbance was measured each minute during 6 min and the differences were used to calculate enzymatic activity of CAT present in sample tissues.

For superoxide dismutase (SOD) activity, 50 µL of EE were added to a reaction mix containing phosphate buffer (50 mM), nitro blue tetrazolium chloride (NBT), methionine and riboflavin. Each reaction tube was vortexed and exposed to uniform light of 12.5 lux for 15 min. Absorbance was read at 560 nm. SOD activity was reported as the amount of enzyme that inhibits the rate of NBT reduction by 50% under the above assay conditions.

Phenylalanine ammonia lyase (PAL) activity was quantified by the presence of cinnamic acid generated by the L-phenylalanine catalysis measured spectrophotometrically at

290 nm. The reaction was made with 100 µL of EE and 100 µL 60 µM/mL L-phenylalanine, incubated at 37 °C for 1 h and stopped by the addition of 50 µL of 1 M trichloroacetic acid.

Finally, total phenolics (PHEN) and flavonoids (FLAV) were determined from methanolic extracts (MEs) of the samples. Tissues were pulverized with liquid nitrogen, lyophilized and mixed with 500 µL of methanol for each 50 mg of sample. The mix was then, sonicated 30 min in an Elmasonic E 30 H sonicator and centrifuged for 15 min by 12,300× *g* at room temperature. Once MEs were ready, PHEN was determined following the assay established by Singleton et al. in 1965, where phenolic compounds react to Folin-Ciocalteu reagent giving place to a blue color in the solution: 40 µL of ME were mixed with 460 µL of distilled water and 250 µL of 1 N Folin-Ciocalteu reagent; after 5 min, 1250 µL of 20% sodium carbonate were added. Solution was vortexed and incubated for 2 h in darkness. Absorbance was measured at 760 nm and results were reported as mg of gallic acid equivalents (GAE). From the same ME, FLAV was measured as following: 50 µL of ME were mixed with 180 µL of distilled water and 20 µL of 2-aminoethyldiphenylborate in each well of a 96-well microplate. The absorbance of the solution was measured at 404 nm and results obtained were expressed as mg of rutin equivalent. This methodology is based on Oomah et al. [27].

Additionally, the content of protein in the same enzyme extract was determined by the Bradford method [28] in order to report enzyme activity by protein content of each sample. All spectrophotometric measurements were accomplished in triplicate in a spectrophotometer multiskan SkyHigh from ThermoScientific. All results were normalized according to the control plants (Vt/V0) and reported as dimensionless data, where Vt is the concentration or enzymatic activity value in samples of elicited plants and V0 an average of values in un-treated plants (controls for each time), as reported earlier [29].

2.5. Data and Statistical Analysis

For each variable in each sampling time, one-way ANOVA was performed to statistically confirm differences between controls and treatments and times with significant difference were evaluated by Tukey test ($p = 0.05$). ANOVA and Tukey test were performed using software JMP version 13.2.0 (JMP statistical discovery Cary, NC, USA).

Normalized data of each variable were used to establish a non-linear regression model, considering as input variables the stage of plants (young or adult) and time after application (hours). As the output variable, plant responses of each variable were considered. Modeling was performed in MATLAB R2022a (The MathWorks Inc.: Natick, MA, USA).

Principal component analyses (PCAs) were used to visualize the distribution of the samples and their variance by treatment, counting all variables. PCAs were performed using the 'prcomp' function in the software R version 4.1.3 (R Core Team: Vienna, Austria) and the 'factoextra' package was used to extract and visualize the output of the PCA (Kassambara and Mundt, 2017).

3. Results

3.1. Plant Immunity Marker Levels by Self eDNA Treatment

Activation of the PPP by the application of self eDNA was observed. Starting with an increment of PAL activity in plants under treatment since 1 h post application (1 HPA), this remained significant until 10 days post-application (10 DPA) (Table 2). As one of the enzymes required in the initial stage of PPP, PAL activation reflected an increment of FLAV and PHEN at 10 days after the treatment (10 DPA) (Table 2 and Figure 2). This increment remained stable until 10 days after the second application (10 DPSA) for PHEN but was no longer significant at 5 days post-second application (5 DPSA) for FLAV. It is important to highlight that these markers showed different behaviors between the first and second applications of the same treatment (Figure 2). Results showed a lower increment of PPP markers as response of the second application than the first applied to young plants; however, PPP responses to the second application of self eDNA showed a significant difference to control plants at 5 DPSA.

Table 2. Tomato immune responses to different eDNA treatments.

TIME	VARIABLE	CONTROL	SELF	MIX	FUSARIUM 150	FUSARIUM 15	LATE SELF
1 HPA	PHEN	0.202	0.241	0.182	0.228	0.284	0.202
	FLAV	3.313	3.425	3.808	3.551	4.844 *	3.313
	HP	0.003	0.008 *	0.010 *	0.007 *	0.003	0.003
	PAL	253.149	454.699 *	363.757 *	464.851 *	133.536 *	253.149
	SOD	1.78×10^{-4}	3.19×10^{-4} *	1.29×10^{-6} *	4.54×10^{-5} *	5.08×10^{-5} *	1.78×10^{-4}
	CAT	3.145	65.462 *	48.141 *	78.179 *	57.957 *	3.145
5 HPA	PHEN	0.219	0.210	0.223	0.219	0.309 *	0.219
	FLAV	4.289	4.016	4.163	4.063	4.691	4.289
	HP	0.004	0.004	0.004	0.004	0.003	0.004
	PAL	183.561	394.401 *	436.755 *	249.234	128.307	183.561
	SOD	2.17×10^{-7}	1.69×10^{-4} *	1.1×10^{-4} *	8.06×10^{-7}	7.25×10^{-6}	2.17×10^{-7}
	CAT	4.915	64.325 *	78.921 *	76.581 *	75.060 *	4.915
1 DPA	PHEN	0.222	0.207	0.219	0.241	0.192	0.222
	FLAV	3.505	4.020	3.776	2.820	4.013	3.505
	HP	0.006	0.009	0.005	0.004	0.005	0.006
	PAL	193.512	519.680 *	308.430 *	260.832 *	144.734 *	193.512
	SOD	1.83×10^{-4}	3.19×10^{-5} *	3.24×10^{-7} *	5.46×10^{-6}	1.95×10^{-6}	1.95×10^{-6}
	CAT	3.667	60.252 *	47.908 *	79.210 *	83.077 *	3.667
5 DPA	PHEN	0.217	0.237	0.323 *	0.219	0.156	0.217
	FLAV	3.585	3.760	3.600	2.369 *	3.467	3.585
	HP	0.001	0.012 *	0.003	0.000	0.005	0.001
	PAL	186.810	453.329 *	273.590 *	241.233	188.490	186.810
	SOD	3.6×10^{-4}	2.5×10^{-4} *	4.11×10^{-6} *	7.16×10^{-7}	1.45×10^{-6}	1.45×10^{-6}
	CAT	8.846	54.872 *	49.055 *	80.364 *	94.675 *	8.846
10 DPA	PHEN	0.156	0.332 *	0.152	0.266 *	0.162	0.156
	FLAV	3.771	5.839 *	2.833 *	4.693 *	3.710	3.771
	HP	0.008	0.012	0.009	0.002	0.007	0.008
	PAL	230.104	449.059 *	171.834	449.639 *	318.652 *	230.104
	SOD	3.67×10^{-7}	2.4×10^{-4} *	3.29×10^{4} *	1.81×10^{-6}	1.65×10^{-7}	9.23×10^{7}
	CAT	13.957	64.675 *	40.184 *	7.903	3.617	13.957
1 HPSA	PHEN	0.256	0.464 *	0.180	0.329	0.315	0.247
	FLAV	4.681	6.478 *	3.765	5.131	4.519	3.781
	HP	0.015	0.020	0.022	0.055 *	0.012	0.028
	PAL	356.371	707.890 *	732.710 *	583.202 *	353.717	336.848
	SOD	1.13×10^{-6}	3.17×10^{-7}	5.95×10^{-5}	6.04×10^{-6} *	1.85×10^{-7}	1.53×10^{-6}
	CAT	14.645	5.486	9.027	22.044	28.959 *	20.549
5 DPSA	PHEN	0.271	0.419	0.276	0.393	0.712 *	0.277
	FLAV	5.096	4.472 *	4.327 *	5.170	5.138	8.291 *
	HP	0.002	0.012	0.014 *	0.036*	0.007	0.021 *
	PAL	325.189	436.558 *	828.892 *	360.748	266.180 *	351.545
	SOD	1.42×10^{-6}	7.59×10^{-7}	4.72×10^{-7}	3.10×10^{-6}	4.99×10^{-6}	1.54×10^{-4} *
	CAT	12.187	6.006	21.231 *	4.117 *	13.941	17.168
10 DPSA	PHEN	0.260	0.420 *	0.552 *	0.386	0.317	0.297
	FLAV	5.786	4.600 *	7.119 *	5.067	6.746 *	9.614 *
	HP	0.005	0.003	0.002	0.007	0.018 *	0.027 *
	PAL	281.315	456.569 *	997.859 *	364.336 *	760.619 *	288.871
	SOD	5.14×10^{-5}	2.8×10^{-4} *	1.1×10^{-5}	2.67×10^{-7}	1.49×10^{-7}	1.78×10^{-6}
	CAT	12.524	5.937	1.072 *	4.572	16.041	22.765 *

Variables and treatments have been abbreviated as follows: PHEN = total phenols (mg of gallic acid equivalents), FLAV = flavonoids (mg of rutin equivalent), HP = hydrogen peroxide (nmol/L), PAL = phenylalanine ammonia lyase activity (U/mg of protein), SOD = superoxide dismutase activity (U/mg of protein), CAT = catalase activity (U/mg of protein). SELF = *S. lycopersicum* eDNA, MIX= mixed plants eDNA, FUSARIUM150 = *F. oxysporum* eDNA at 150 ppm and FUSARIUM15 = *F. oxysporum* eDNA at 15 ppm. Asterisk (*) indicates significant difference in comparison to control plants by Tukey test ($p = 0.05$).

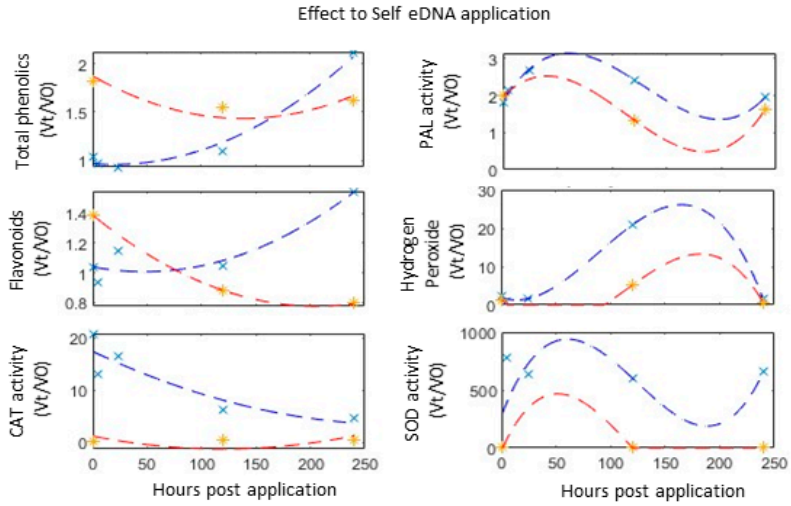


Figure 2. Mathematical Modeling of plant immune responses to self eDNA. Data showed in normalized dimensionless values for each sampling time in hours post-treatment application. Blue lines represent responses to first application and red lines represent responses to second application of treatment.

Regarding the presence of HP and the activities of antioxidant enzymes CAT and SOD as response to application of self eDNA in tomato plants, an early activation of the three markers was observed. This activation remained significant until 10 DPA for SOD and CAT but the presence of HP was significantly different only at 1 HPA. Interestingly, a second significant increment in HP was observed at 5 days after both applications (5 DPA and 5 DPSA) (Figure 2).

Figure 3 show the responses of immunity markers in tomato plants treated with self eDNA at 50 ppm only at 45 days after transplant. As observed, all responses at the early stage (blue line) remained inactivated for this treatment, because no treatment was applied. In this case, results differed greatly from the early application of self eDNA. Activation of immunity markers was lower in adult plants; in fact, a statistical difference between controls and this treatment was only observed in the increment of HP and FLAV at 5 and 10 days post application and CAT activity at 10 DPA (Table 2).

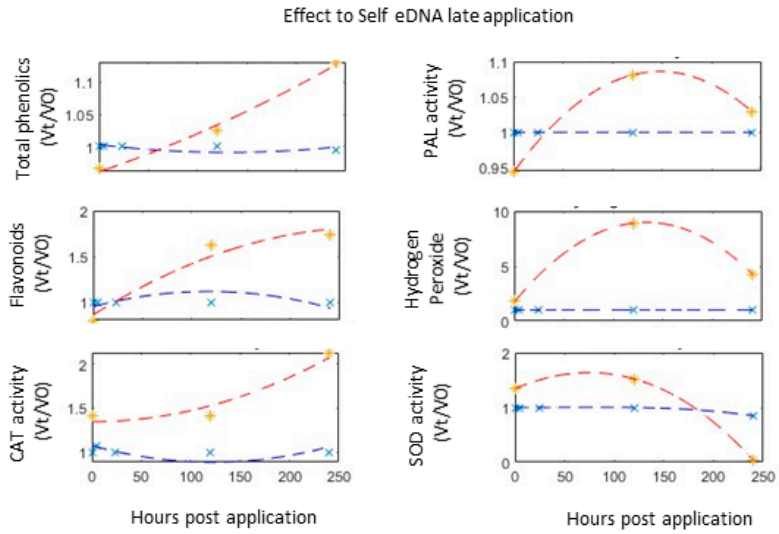


Figure 3. Mathematical Modeling of plant immune responses to self eDNA late application. Data showed in normalized dimensionless values for each sampling time in hours post-treatment application. Blue lines represent responses to first application and red lines represent responses to second application of treatment.

3.2. Plant Immunity Markers Levels by Non-Self eDNA Treatment

No-conspecific DNA was also evaluated as non-self eDNA treatments. eDNA extracted from a known tomato pathogen (*Fusarium oxysporum*) was tested in two different concentrations to evaluate its suggested role as a pathogen-associated molecular pattern (PAMP) for the plant immune system (Figure 4). When treated with 150 ppm *Fusarium* eDNA, tomato plants showed an intense response of the antioxidant system, especially CAT activity, with a more than 20-fold increase in the first hours after treatment until 5DPA. SOD activity showed a lower but significant activation that remained stable until 10DPA. In contrast, the presence of HP was not significantly detected with this treatment. Analyzing the response of these markers to the second application of 150 ppm *Fusarium* eDNA, the detected values of HP and SOD activity even more intense than the response to the first application (Figure 4). Additionally, when *Fusarium* eDNA was applied at lower concentration (15 ppm), the markers of the antioxidant system showed higher responses and displayed similar activation rates for the first and second application (Figure 5). Although the responses were more intense, it was shown that that the signals were back to normal values (no different than controls) at 10 DPA (Figure 5, Table 2). PPP markers showed low responses to *Fusarium* eDNA at both concentrations, having the higher concentration/activity at the last evaluated point for the second application (10 DPSA, Figures 4 and 5). Here, more evaluation times are needed to characterize this latter immune response.

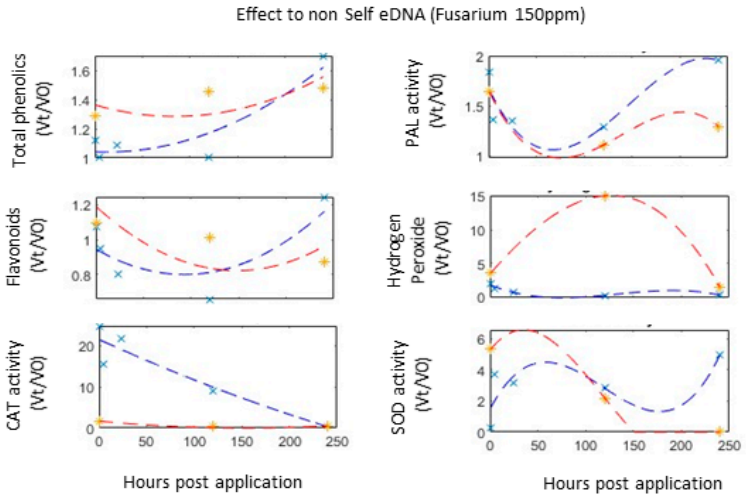


Figure 4. Mathematical modeling of plant immune responses to non-self eDNA (*Fusarium* eDNA at 150 ppm) applied in adult plants. Data showed in normalized dimensionless values for each sampling time in hours post treatment application. Blue lines represent responses to first application and red lines represent responses to second application of treatment.

Finally, the application of non-self eDNA from a mix of plants was evaluated. Table 2 shows that, in fact, there was activation of some of the evaluated variables. Similar to the responses to pathogen eDNA, PPP markers displayed low but incremental responses, with their higher level at the last sampling time as a response to the second application of the treatment (Figure 6). In contrast, CAT and SOD activity showed high activation (more than 15-fold and 500-fold) early post-first application. Nevertheless, the responses of the evaluated immune markers to non-self eDNA in the evaluated times were lower than those obtained for self eDNA.

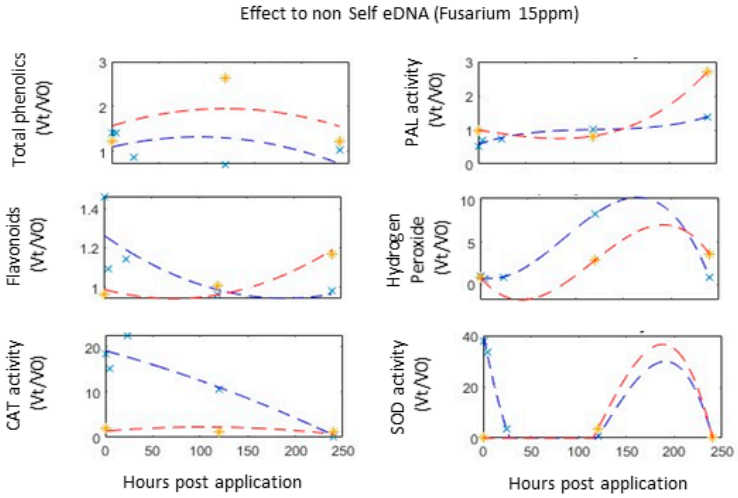


Figure 5. Mathematical modeling of plant immune responses to non self eDNA (*Fusarium* eDNA at 15 ppm) applied in adult plants. Data showed in normalized dimensionless values for each sampling time in hours post treatment application. Blue lines represent responses to first application and red lines represent responses to second application of treatment.

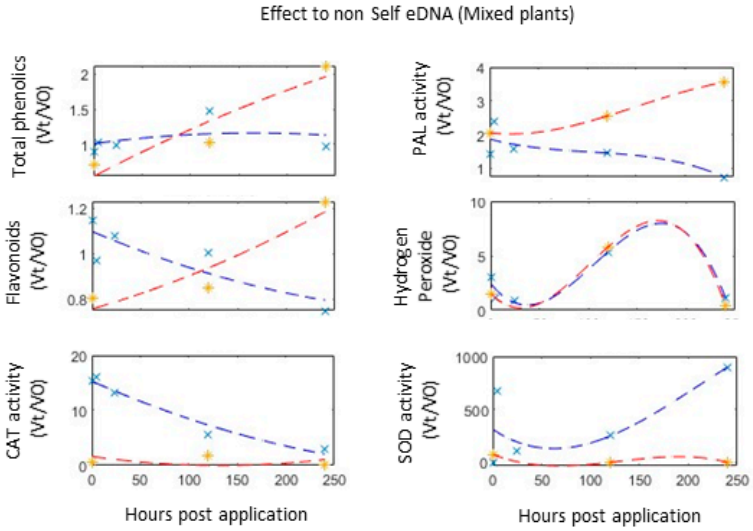


Figure 6. Mathematical modeling of plant immune responses to non self eDNA (mixed plants eDNA) applied in adult plants. Data showed in normalized dimensionless values for each sampling time in hours post treatment application. Blue lines represent responses to first application and red lines represent responses to second application of treatment.

3.3. Principal Component Analysis of Plant Immune Responses to eDNA Treatments

A principal component analysis (PCA) was made for all variable responses (Figure 7). The first two components obtained explained 80.97% of the maximal variance among all data. Analysis resulted in a slight differentiation of individuals by treatment. On the one hand, a group of individuals related to SOD activity conformed to mixed plants eDNA treatment at all evaluated times, except for the first and last sampling time. Another group related to CAT activity was conformed to mainly by *Fusarium* eDNA treated plants at both evaluated concentrations. Finally, there were some individuals partially grouped and related to PPP markers that included self eDNA, *Fusarium* eDNA and mixed eDNA treated plants (Figure 7).

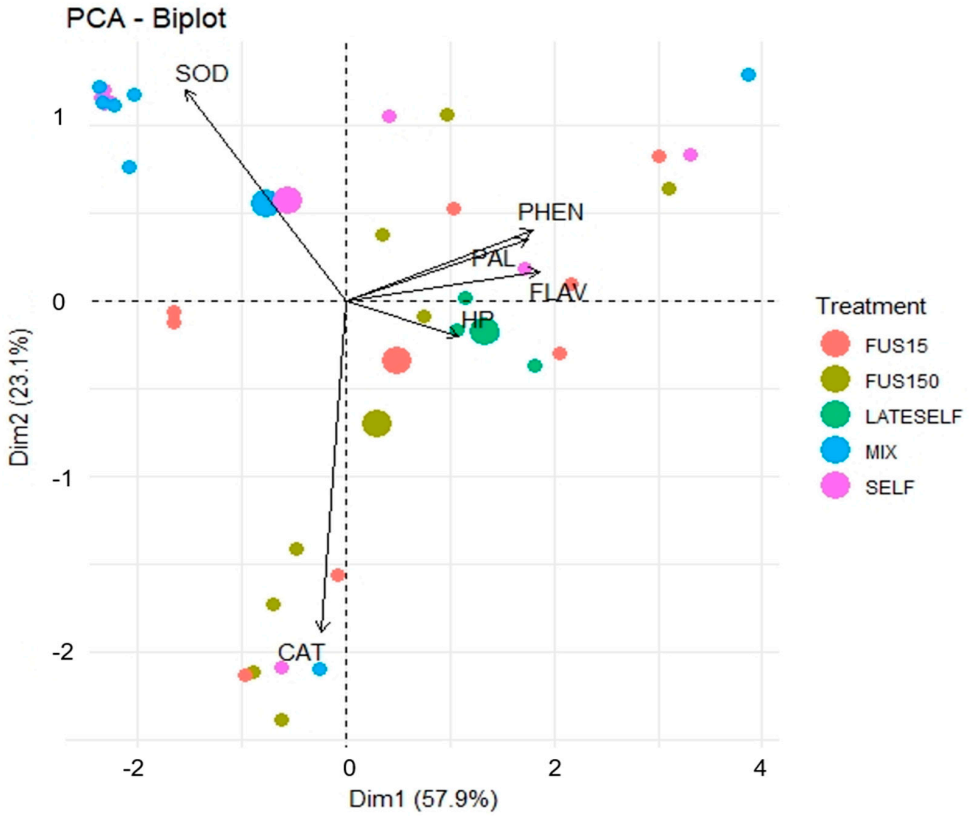


Figure 7. Dispersion of samples colored by treatment and the correlation of the evaluated variables for all treatments. Maximal variance explained by each principal component given in brackets.

For the measured variables, a circle of correlation was obtained, where variables PAL, PHEN and FLAV were strongly correlated. These variables presented high correlation with HP but no correlation with SOD and CAT (Figure 7). PCAs of each variable were also performed in order to present the dispersion of each sampling time and more than 80% of each variable variance was explained by the two first principal components (data not shown). Interestingly, correlation circles of all PCAs displayed a strong correlation between PPP markers and weak correlation with SOD and CAT activities.

Additionally, in Figure 7 a slight grouping of the samples was observed showing similar responses to self and mixed plants eDNA. This can likely be explained by the phylogenetic origin of the DNA applied in those treatments.

4. Discussion

As a strategy to maintain their own homeostasis, plants have developed the ability to analyze the near environment through the recognition of environmental molecules and by sensitizing their immune system in response to signals that may represent danger. Previously, these signals have been termed eustressors and classified as elicitors if they have a biological nature [2,30]. Related to this, the natural effect of "priming" has been described as a more effective, faster and more intense response to a previously receipted signal (or eustressor); therefore, this response represents a lower fitness cost than the first [31,32]. For the priming process to happen an early signal must be presented to the plant immune system for the activation of immune pathways and accumulation of immune metabolites; in this way, the second recognition of danger signals must activate primed responses. One example of priming defenses is the induced systemic resistance or ISR caused by the microbiota that colonize the plant [33]. From an agronomical perspective, elicitors represent a potentially sustainable way to enhance or protect several aspects of crops when applied in a controlled way.

The eDNA has been identified as an effective elicitor capable of activating defense responses and plants have showed the ability to respond differently depending on the source of the DNA. Nonetheless, few works have explored this effect from a productive point of view to clarify specific aspects of its behavior and its characterization through time. In this work, we described the behavior of six different immune markers in tomato plants responding to different eDNA treatments in two phenological stages. In a simplistic way, we grouped the evaluated variables into two defense systems: phenylpropanoid pathway (PPP) and antioxidant enzyme system to simplify the description of results.

As expected, self eDNA treatment showed a higher response for both PPP and antioxidant markers; however, non-self eDNA responses were also significant. The observed differences in responses to self and non-self eDNA treatments may be the result of a remarkable differential gene expression as reported by Chiusano et al. [13] and, therefore, likely eDNA might play different roles according to its origin as a PAMP, DAMP, etc.

In addition, each treatment was applied twice (except in the late application of self eDNA), expecting to identify any signature of priming (although no transgenerational effects were studied in this work) in the time and intensity of defense response. However, only few immune markers seem to behave in this way. This could be due to the time between applications, as priming has been reported mostly in shorter times between two stimuli [34]. In general, responses to eDNA showed priming characteristics in responses to some variables such as HP, CAT activity and FLAV for self Edna (Figure 2), PHEN, PAL activity and HP for *Fusarium* eDNA (Figures 4 and 5) and PPP markers for mixed eDNA (Figure 6).

Additionally, we showed a clear difference in intensity of responses to self eDNA depending on the stage of application (Figures 2 and 3). Data suggested that adult plants have been exposed to several environmental molecules through their lifetime (including self and non-self eDNA) and have a higher response threshold. With less sensible responses, as in the case of non-self eDNA treatments, the stimulus must be higher to obtain the same results as those responding to an early application [35].

Although the goal of the assays presented here was not to prove the efficiency of an activation of the immune system by eDNA through pathogenesis tests in pathogen–tomato interactions, we can suggest that the results of this kind of interactions may be altered by the production of metabolites directly related to plant defense as flavonoids, as a consequence of the activation of specific metabolic routes by eDNA application [7,11,17]. Pathogenesis tests in future research need to be performed in order to confirm the efficacy of the immune system activation against real infections.

When we are able to understand more about the behavior of plant responses to eDNA, it becomes possible to design strategies of controlled elicitation using this type of elicitor for agricultural crops, not only biologically efficient, but also economically viable for producers. Currently several elicitors are being explored for the development of the new generation of agricultural treatments [35]. In the present work, we report that almost all the evaluated variables maintained activation until day 10 after one eDNA application; however, more sampling times are needed to determine the costs and benefits of the elicitation.

As shown in the results, the behavior of the immune markers evaluated was highly variable as part of a complex web of signal transductions that form the defense response in plants. In an effort to determine which variables bring out more information on plant immune responses, a principal component analysis and correlation circles of the evaluated variables with similarities between each treatment were carried out. In general, PPP markers displayed a strong correlation between themselves and weak correlation to CAT and SOD activities. This information provides mathematical support for the design of a rational strategy to evaluate future treatments, expecting the activation of a plant's immune system when designing controlled elicitation strategies for sustainable agriculture using eDNA treatments. Additionally, it will be highly important to consider the phenomenon of hormesis when studying this type of strategy.

5. Conclusions

In this paper we present six variable responses to five different eDNA treatments in tomato plant. These variables have been selected based on their close relation to immune response in plants and simplicity of measurement. Data were collected at different times after the application of treatments and mathematical modeling of the behavior for each response had been performed. A significant activation of every variable has been observed at different times for each treatment, but responses to self eDNA compared to non-self eDNA were generally higher (up to 10-fold) and longer, as some remained significant until last day of sample after the application (10 days). Although more variables are needed to be explored, the application of self eDNA has been suggested as a potential agricultural treatment to reduce the incidence of pests in vegetal crops. Being from a natural source, its use can also be applied in organic productions and meanwhile can be obtained from the same crop biological waste, promoting a sustainable production system.

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