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Extracellular self-DNA induces local inhibition of growth, regulates production of reactive oxygen species, and gene expression in rice roots

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Abstract

The capacity of extracellular self-DNA (esDNA) to inhibit growth is getting more research attention as this could be explored for several purposes, including the development of specific bioherbicides. While the inhibitory effect has been studied in several dicotyledon species, little is known about the effects and subsequent signaling processes in monocots. Here, we measured the growth, counted the number of lateral and crown roots, determined greenness index, quantified the production of $O_2^{\cdot-}$ and H_2O_2, and determined the expressions of genes encoding antioxidant enzymes (*SOD*s and *CAT*s) in rice (*Oryza sativa* L.), a model plant of monocots. After 7 d of germination, rice roots were exposed to 0, 75, and 150 μg cm^{-3} of esDNA. Inhibitory effect was found to be negatively correlated to esDNA concentration, as indicated by the length of primary roots. Interestingly, this negative effect was only observed in the directly exposed organ (root) but not in the length of shoot or fresh mass of the whole seedling. The percentage of greenness index of leaves and number of crown and lateral roots were also similar across treatments. However, esDNA exposure to root increased production of $O_2^{\cdot-}$ and H_2O_2 in the root. At the molecular level, the response was characterized by the decreased expression of the antioxidant genes *SOD*3, *CAT*B, and *CAT*C. These findings suggest that esDNA inhibits rice growth locally in, *e.g.* in treated roots, and the responses involve increased production of ROS and suppression of antioxidants. This study could be the basis for determining the combination of concentration and period of exposure that might significantly inhibit total growth of monocot weeds with a minimum effect on the crop.

Keywords: bioherbicide, CAT, DAMP, esDNA, SOD.

Introduction

The growing global population demands a rapid increase in the food supply. However, the ecological costs are incalculable to tolerate the excessive use of chemicals and constant conversion of nature to farming land. Alternative means of green and efficient farming are required to support the population and protect the environment. The application of plant-based elicitors and biostimulants has been shown to obtain improved immune responses, enhancement of plant growth, and an increase in protein content (Carbajal-Valenzuela *et al.* 2021). Recent studies (Mazzoleni *et al.* 2015, Cartení *et al.* 2016, Gallucci and Maffei 2017) demonstrated that extracellular self-DNA (esDNA) recognition is conserved across the tree of life that serves as a prominent danger sensing mechanism, leading to augmented defense, such as inhibited growth or modification in metabolism. This pattern is a potential alternative that can be explored as a plant-based general biostimulant to replace chemically synthesized fertilizers or pesticides (Ferrusquía-Jiménez *et al.* 2020, Meitha *et al.* 2021). Furthermore, extracellular DNA is considered as an important signal molecule in crop protection that enhances the possibility of a circular economy system (Carbajal-Valenzuela *et al.* 2021).

In particular, the role of esDNA as a damage-associated molecular pattern (DAMP) in plants has been proposed by several studies, and systematically reviewed by Ferrusquía-

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Abbreviations: CAT - catalase; DAB - diaminobenzidine; DAMP - damage associated molecular pattern; dpt - days post treatment; esDNA - extracellular DNA; NBT - nitroblue tetrazolium; PAL - phenylalanine ammonia lyase; PAMP - pathogen associated molecular pattern; ROS - reactive oxygen species; SOD - superoxide dismutase.
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Jiménez *et al.* (2020). The external presence of molecules that normally reside in the cellular interior could signal danger to the healthy cells, informing damage, or attack by pathogens/herbivores/insects. This signal will be responded to by activating the immune system. Matzinger's danger model emphasizes that any successful immune response depends on the detection of "endogenous danger signals", a continuous surveillance for integrity or damage (Matzinger 1994). It has been discovered that the presence of esDNA in the root tip of *Pisum sativum* improved resistance to the fungal pathogen *Nectria haematococca* (Wen *et al.* 2009). Exposure to fragmented esDNA induced responses related to immunity, such as plasma membrane potential depolarization and Ca^{2+} signaling in lima bean and maize (Barbero *et al.* 2016) as well as ROS generation and the activation of MAPKs in common bean (Duran-Flores and Heil 2018). However, growth inhibition response was also induced upon esDNA application as recorded in *Acanthus mollis* (Mazzoleni *et al.* 2015), *Lactuca sativa* (Vega-Muñoz *et al.* 2018), *Phaseolus vulgaris* (Duran-Flores and Heil 2018), and *Arabidopsis thaliana* (Chiusano *et al.* 2021). These studies highlighted limited root growth, with an emphasis on physiological parameters such as chlorotic leaves and necrotic root tips (Chiusano *et al.* 2021). Thus, the response to esDNA exposure could involve a trade-off that growth was stalled until the level of sensed damage decreased; or lead to death if the damage signal was constantly present at a threatening level.

Crops, especially rice, maize, and wheat have contributed enormously to global food security over the last half-century. Innovative research on these three most important cereal grains could be the solution to provide food for the 800 million people still living in chronic hunger and to sustain food production in the face of climate change (Kropff and Morell 2018). To date, only two studies on monocot responses upon exposure to extracellular DNA are recorded but none of them included any measurement of growth inhibition. The study on *Zea mays* (Barbero *et al.* 2016) emphasized on plasma membrane depolarization upon self esDNA treatment. While the study on *Triticum aestivum* (Le Mire *et al.* 2019), explored induced immunity following the application of non-self exDNA treatment [CpG ODN (oligodeoxynucleotides)] from *Zymoseptoria tritici*). Furthermore, the measurement of total fresh mass or shoot growth was not available. These are important parameters to understand whether growth inhibition was induced locally or systemically in the whole plant as it eventually affects the yield.

Reactive oxygen species (ROS) are key players in plant stress signaling (Baxter *et al.* 2014), an essential in redox biology to direct cells toward stress acclimation (Mittler 2017). Barbero *et al.* (2021) suggested that ROS modulation was among the early responses, within 30 min, following exposure of esDNA to *Solanum lycopersicum*. Within 1 h of esDNA recognition by *A. thaliana* cells, differentially upregulated expression of *alternative oxidase* 1d and two transcription factors related to ROS were demonstrated (Chiusano *et al.* 2021). In *Phaseolus vulgaris,* increased hydrogen peroxide content was recorded at 2 h post-application of esDNA (Duran-Flores and Heil 2018). After 5 d, esDNA exposure elevated the relative expression of antioxidant genes (*superoxide dismutase/SOD, catalase/CAT,* and *phenylalanine ammonia lyase/PAL*) in *Lactuca sativa* (Vega-Muñoz *et al.* 2018). These studies suggested that ROS was involved in the subsequent signaling triggered by esDNA. However, the data on how ROS modulation, balanced by the antioxidant system, correlates to phenotypic changes at a one-time point is not yet available.

In this study, we analyzed the growth of rice (*Oryza sativa* L. cv. MSP13) as a model for monocot plant, regulations of ROS, and expressions encoding antioxidants following exposure to esDNA at a range of concentrations. More evidence of H_2O_2 regulation was documented in the literature, including the quantification in the treated samples (Duran-Flores and Heil 2018) and the expression of two genes (*SOD* and *CAT*) that immediately balance H_2O_2 accumulation (Vega-Muñoz *et al.* 2018). Thus, to gain a further understanding, we determined H_2O_2 production, superoxide anion ($O_2^{\cdot-}$) as the precursor species, and gene expression of three *SOD* isoforms (*OsSOD Cu/Zn, OsSOD*3 *Cu/Zn, OsSOD*4 *Cu/Zn*) and three *CAT* isoforms (*OsCAT*A, *OsCAT*B, *OsCAT*C).

Materials and methods

Plant material and preparation of esDNA: Rice seeds (*Oryza sativa* L. cv. MSP13) were grown for 4 - 6 weeks on the soil at room temperature with a approximately 12-h photoperiod and daily watering. Prior to genomic DNA extraction, the plants were dipped in liquid nitrogen. DNA extraction was performed according to modified cetyltrimethylamonium bromide (CTAB) plant DNA extraction protocol originated from Doyle and Doyle (1987) and Cullings (1992) as follows. The plant materials were ground in a mortar and pestle aided with the liquid nitrogen. For each 6 g of the plant fine powder, 15 cm^3 CTAB extraction buffer [2 % (m/v) CTAB, 2 % (m/v) polyvinylpyrrolidone (PVP), 0.2 % (v/v) β-mercaptoethanol, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0)] was added in a 50 cm^3 conical centrifuge tube. The homogenate was then incubated at 65 °C for 40 min while being mixed intermittently using a vortex mixer within every 10 min break. Then, 1 volume (15 cm^3) of ice-cold chloroform/isoamyl alcohol (24:1) solution was added into the lysate and mixed. The mixture was centrifuged for 10 min at 4 °C and 15 000 *g* using *Eppendorf 5804R* refrigerated centrifuge with *FA-45-6-30* rotor (*Eppendorf*, Hamburg, Germany). The clear upper aqueous phase was transferred into a fresh 50 cm^3 conical centrifuge tube. Then, 1 volume (15 cm^3) of ice-cold isopropanol and 1 volume (15 cm^3) of ice-cold 5 M ammonium acetate were added and mixed. The solution was then incubated at -20 °C for 1 h and centrifuged for 20 min at 4 °C and 15 000 *g*. The supernatant was discarded. Then, 5 cm^3 of ice-cold 70 % ethanol was added into the tube to wash the pellet and mixed. The solution was centrifuged for 10 min at 4 °C and 15 000 *g*, followed by the decantation of the supernatant. The pellet was air-dried for 15 min ensuring

the alcohol was completely removed, and then eluted with 400 mm^3 of nuclease-free water.

Following this, fragmentation of genomic DNA was done by sonication at 20 kHz with 30 % power and 0.2 pulse condition for 12 min in *Omni Sonic Ruptor 4000* ultrasonic homogenizer (*Omni International*, Kennesaw, USA). The purity and concentration of genomic and fragmented DNA were determined by measuring the absorbance ratio of 260/280 nm in a *NanoDrop™ Lite* spectrophotometer (*Thermo Scientific*, Waltham, USA). Gel electrophoresis was done to determine the quality of genomic DNA and fragmented DNA [2 % (m/v) agarose gel]. Once the concentration was determined, fragmented DNA was diluted with nuclease-free water into concentrations of 75 µg cm^{-3} and 150 µg cm^{-3} for the respective treatments of esDNA.

Application of esDNA: Rice seeds were soaked in water for 24 h and germinated on plastic containers covered with wet paper towels for 5 d at room temperature with approximately 12-h photoperiod. Moisture was maintained by daily spraying. The roots of seedlings were then immersed in 75 or 150 µg cm^{-3} esDNA (0 µg cm^{-3} served as the non-treated control), and grown in wet paper towel rolls (125 biological replications per treatment). Following this, the seedlings were kept at room temperature and approximately 12-h photoperiod for 7 d.

Determination of growth and ROS production in root: Total fresh mass and primary root and shoot length of each seedling on day 0 of the treatment and day 7 post treatment were measured. Primary roots were already present on day 0 of the treatment, hence only these roots were considered for length measurement. The number of secondary roots (lateral and crown roots) of each seedling was counted only on day 7 post treatment.

$O_2^{\cdot-}$ content and H_2O_2 content in roots were assayed semi-quantitatively by staining with nitroblue tetrazolium (NBT), according to Kumar *et al.* (2014), and 3,3'-diaminobenzidine (DAB), according to Daudi and O'Brien (2012), respectively. NBT will react with $O_2^{\cdot-}$ to form a dark blue insoluble formazan (Kumar *et al.* 2014), while DAB is oxidized by H_2O_2 producing reddish brown precipitate (Daudi and O'Brien 2012). In this study, 20 rice seedlings from each treatment group were used for histochemical staining. Each 10 of them were stained with NBT and DAB, respectively. The whole seedlings were immersed in the respective staining solution and then vacuum infiltrated for 10 min. Prior to immersing in the stain solution, the roots were left intact to prevent additional $O_2^{\cdot-}$ and H_2O_2 production due to wounding during the procedure. Following this, the seedlings were incubated in the staining solution for 5 h at room temperature with a gentle agitation on the rocking shaker at 80 - 100 rpm. Destaining was done in several rounds of washing using 95 % (v/v) ethanol. The images of stained plants were captured by using *Canon EOS 1300D* (*Canon*, Tokyo, Japan) and were also analyzed under *Nikon SMZ745T* dissecting microscope (*Nikon*, Tokyo, Japan). Quantification of the NBT- or DAB- stained root areas was made by using the image analysis software *ImageJ* (Abràmoff *et al.* 2004). The minimum threshold was set at 0 and the maximum at 70 for all of the quantification of stained area. The percentage of stained area was then calculated as the ratio of histochemically stained root pixel area to the total root pixel area of an individual primary root.

The greenness of leaves was evaluated through determination of Greenness Index (GI) based on the photograph of 10 seedlings subjected with each treatment. GI was determined by measuring the Red Green Blue (RGB) value of 15 × 15 pixel area (225 pixels) of each individual leaf using the *Lockable Color Picker* tool of free software *Raw Therapee* as in Meitha *et al.* (2022).

Relative quantification of genes coding antioxidant enzymes: The primers used in this study for amplifying *OsSOD-Cu/Zn, OsSOD*3*-Cu/Zn, OsSOD*4*-Cu/Zn, OsCAT*A*, OsCAT*B*, OsCAT*C, and *UBQ*10 genes were designed by Vighi *et al.* (2017). All the primers were confirmed through amplicon sequencing (*Macrogen Asia Pacific*), and the *BLAST* results can be found in Table 1 Suppl. and Table 2 Suppl.

For the root relative gene expression analysis, 100 rice roots from each treatment group were used and 20 rice roots were pooled for each RNA extraction reaction. Total RNA extraction (5 biological replications per treatment) was conducted using *TRIsure*™ (*Bioline*, Luckenwalde, Germany) by following the manufacturer's manuals. The RNA quality and quantity were checked using *NanoDrop*™ *Lite* spectrophotometer (*ThermoScientific*, Waltham, USA) and gel electrophoresis (Fig. 1 Suppl.). The cDNA synthesis was conducted using *ReverTra Ace*™ *qPCR RT Master Mix* with gDNA remover (*Toyobo*, Osaka, Japan) according to the manufacturer's manuals using 1 µg of total RNA in each reaction. The *SYBR Green* qPCR was conducted using *Thunderbird*™ *SYBR*® qPCR mix (*Toyobo*) with *Quantstudio 1* real-time PCR system (*Thermo Scientific*). The total reaction volume was 10 mm^3 containing 1 × of *Thunderbird*™ *SYBR*® qPCR mix, 0.5 µM of each forward and reverse primer, 0.1 × of ROX™ as the passive reference dye and 1 mm^3 cDNA as the template. The amplification conditions were set as follows: pre-denaturation at 95 °C for 10 s, followed by 40 repeat cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 30 s, and extension at 72 °C for 45 s, with fluorescence reading set in this stage. The melting curve was made through a thermal gradient of 0.1 °C s^{-1} from 60 °C until 95 °C. The internal control used in this experiment was *UBQ*10 gene, and the genes of interest in this experiment were *OsSOD-Cu/Zn, OsSOD*3*-Cu/Zn, OsSOD*4*-Cu/Zn* and *OsCAT*A, *OsCAT*B, *OsCAT*C. The relative gene expression analysis was conducted using the delta-delta CT method according to Schmittgen and Livak (2008).

Statistical analyses: Statistical analyses were conducted using one-way *ANOVA* to compare the means between the non-treated group and treated groups on the 5 % level of significance using *R version 4.1.0* (*R Core Team 2021*) and

visualized using *R/ggplot2* (Wickham 2016). Duncan's multiple range test was used for post-hoc analysis on the 1 % level of significance when *ANOVA* revealed significant differences using *R/Agricolae* (*R Package Version 1.4.0.*; Mendiburu and Yaseen 2020). Linear regression and Pearson correlation determination between esDNA concentration and physiological parameters was conducted using *IBM SPSS Statistics 23* (*IBM Corp 2015*).

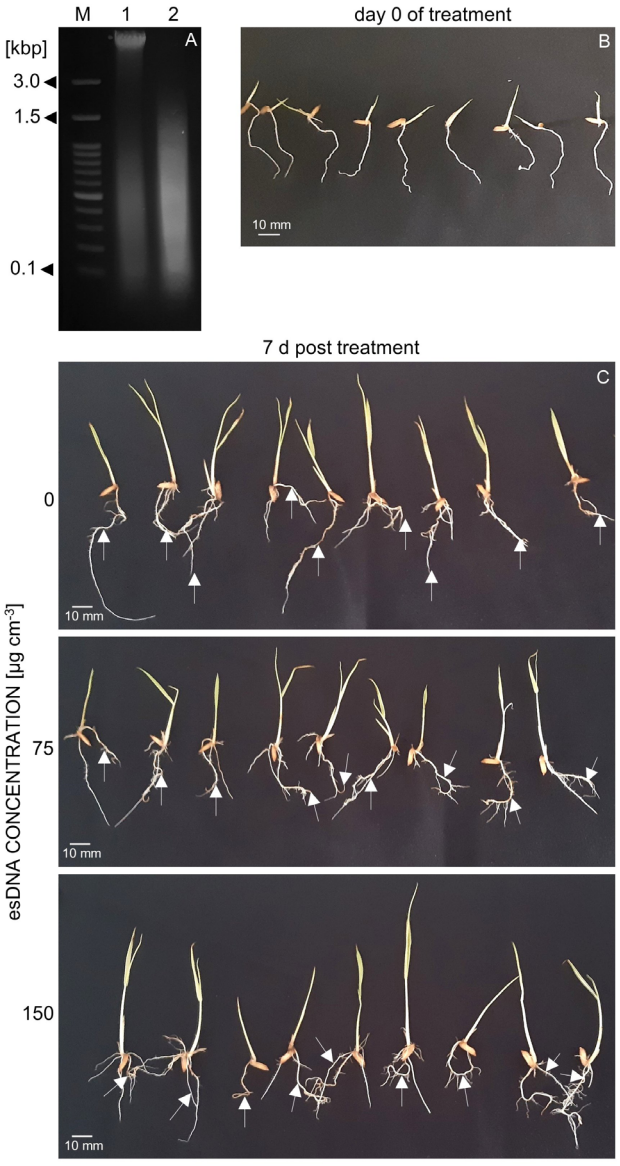

Fig. 1. Preparation of esDNA and treatment to rice (*Oryza sativa* cv. MSP13) seedlings. Genomic DNA was extracted (*A*, *lane 1*) and fragmented to the size of up to 1.5 kbp (*A*, *lane 2*). The seeds were germinated for 5 d prior to esDNA treatments (*B*). On 7 d post-treatment, significantly inhibited ($P < 0.01$) root growth was observed in seedlings treated with 75 and 150 µg cm^{-3} of esDNA (*C*). The presented seedlings were representative of 125 samples from each treatment. Measured primary roots are indicated by *white arrows*.

Results

The result of genomic DNA extraction showed a thick band on the top of the lane with some smear bands detected below (Fig. 1*A*, lane 1). Spectrophotometry analysis of $A_{260/280}$ resulted in a ratio of 1.83, indicating that the extracted DNA was free of protein or RNA contamination. Hence, the smear bands could be a result of unexpected DNA degradation during the extraction process. Following sonication (Fig. 1*A*, lane 2) the fragment sizes ranged up to approximately 1.5 kbp, and intact genomic DNA was no longer detected.

After 5 d of germination, or day 0 of treatment, mean shoot length was 2.033 ± 0.673 cm, primary root length 4.131 ± 0.597 cm, and fresh mass 0.053 ± 0.006 g (Fig. 1*B*). On 7 d post treatment (dpt) (Fig. 1*C*), we re-measured the parameters, evaluated the difference between the obtained values and the initial values, then plotted the increase in root and shoot length and fresh mass (Fig. 2). Inhibition of root growth was evident ($P < 0.01$) and negatively correlated to esDNA concentration (r = -0.69) (Fig. 2*A*). The mean values of increase in primary root length of seedlings treated with 0, 75, and 150 µg cm^{-3} esDNA were 1.453 ± 1.191, 0.481 ± 0.238, and 0.169 ± 0.138 cm, respectively. On the other hand, compared to the non-treated group, a higher increase in shoot length (Fig. 2*B*) and fresh mass (Fig. 2*C*) of the seedlings treated with 75 µg cm^{-3} esDNA was documented. The mean values of increase in shoot length were 2.853 ± 1.894, 3.611 ± 2.182, and 2.490 ± 1.746 cm, while that of increases in fresh masses were 0.029 ± 0.042, 0.034 ± 0.018, and 0.022 ± 0.015 g for seedlings treated with 0, 75, and 150 µg cm^{-3} esDNA, respectively. Similarly, the greenness index of the leaves and number of lateral/crown roots showed no significant difference between water and esDNA treated seedlings.

The accumulation of $O_2^{\cdot-}$ and H_2O_2 in roots were determined semi-quantitatively using NBT (Fig. 3*A*,*C*) and DAB (Fig. 3*B*,*D*) staining, respectively. The mean values of proportion of NBT stained areas were 2.325 ± 1.832, 5.828 ± 2.296, and 5.662 ± 0.694 %, while that proportion of DAB-stained areas were 35.963 ± 7.117, 51.207 ± 7.862, and 50.878 ± 5.069 % in roots treated with 0, 75, and 150 µg cm^{-3} esDNA, respectively. We documented that accumulation of $O_2^{\cdot-}$ and H_2O_2 in either 75 or 150 µg cm^{-3} esDNA treated seedlings was significantly higher compared to their control, although the increase was not in a concentration-dependent manner. The accumulation of $O_2^{\cdot-}$ and H_2O_2 in 75 µg cm^{-3} and 150 µg cm^{-3} esDNA treated seedlings were not distinctive.

Antioxidant system is a key to balancing ROS accumulation, preventing the cell from going through oxidative damage. In this study, the relative expressions of genes encoding antioxidant enzymes *superoxide dismutase* (*SOD*), *SOD*3, *SOD*4, *catalase* (*CAT*)A, *CAT*B, and *CAT*C were measured. Among the three *SOD* isoforms, we found that only *SOD*3 was significantly regulated by esDNA treatment. Compared to control, *SOD*3 expression was reduced in 150 µg cm^{-3} esDNA treated seedlings but not when treated by 75 µg cm^{-3} esDNA. The gene expressions

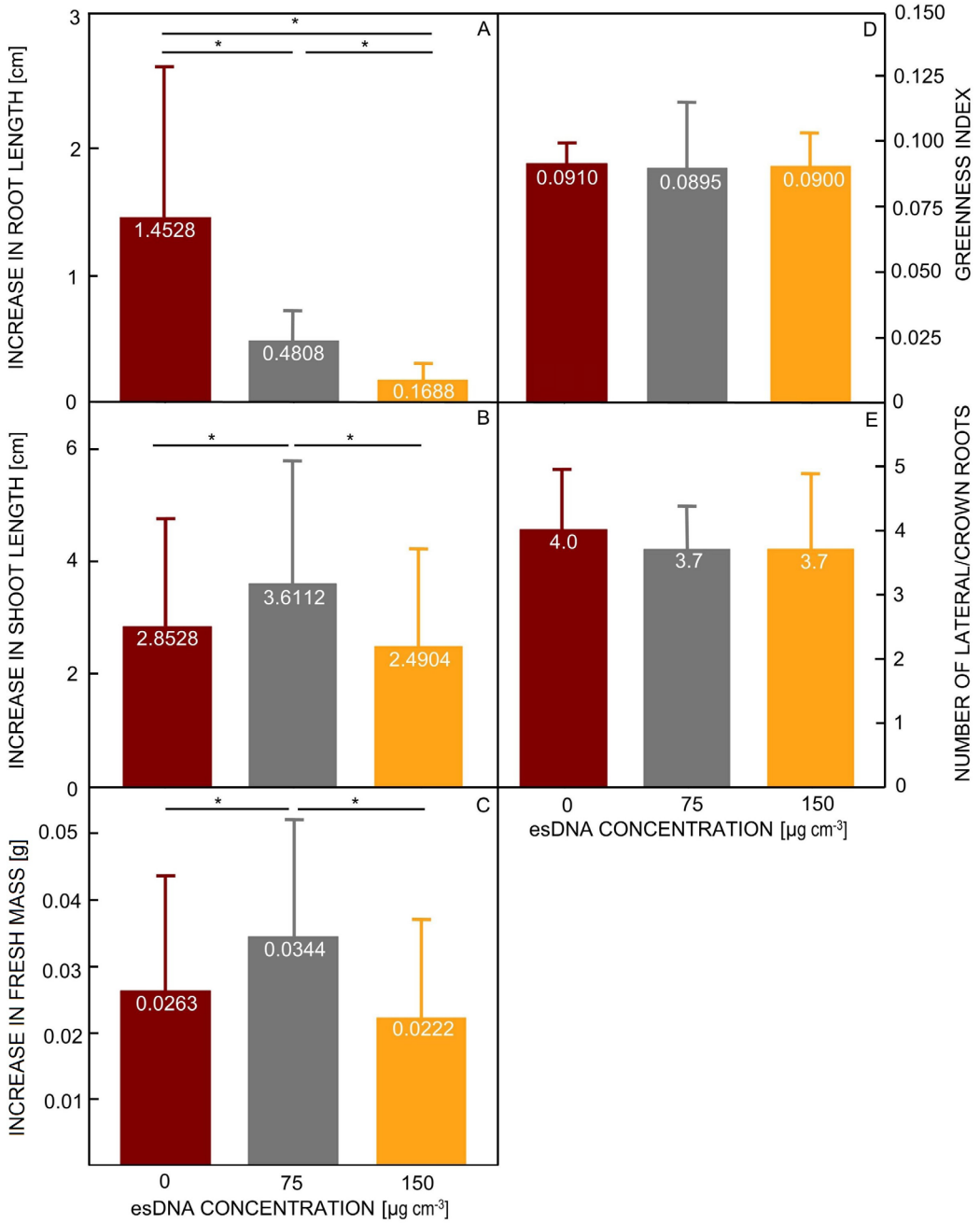

Fig. 2. Phenotypic parameters measured on 7 d post-treatment. The increase in root length (*A*) was negatively correlated to esDNA concentration and significantly different between the treatment groups. However, the responses in shoot length (*B*) and fresh mass (*C*) were not correlated to esDNA concentration. The values in (*A*-*C*) were obtained from 125 treated seedlings in each treatment group. esDNA treatment did not affect the greenness index (*D*) and the number of lateral/crown roots (*E*). The values in (*D*) and (*E*) were obtained from 10 treated seedlings in each treatment group. Statistically significant values (Duncan Multiple Range Test, $P < 0.01$) are indicated by the lines and asterisks above the bars.

of two *CAT* isoforms, *CAT*B and *CAT*C, showed a similar regulation. Thus, the relative genes expression of *SOD*3, *CAT*B, and *CAT*C were significantly suppressed in roots treated with 150 µg cm^{-3} esDNA (Fig. 4).

Discussion

Recent studies have reported that exposure to esDNA elicited immune responses (Duran-Flores and Heil 2018, Barbero *et al.* 2021) and at certain concentrations inhibited growth in plants (Mazzoleni *et al.* 2015, Vega-Muñoz *et al.* 2018, Duran-Flores and Heil 2018, Chiusano *et al.* 2021). This has been attributed to the proposed role of esDNA

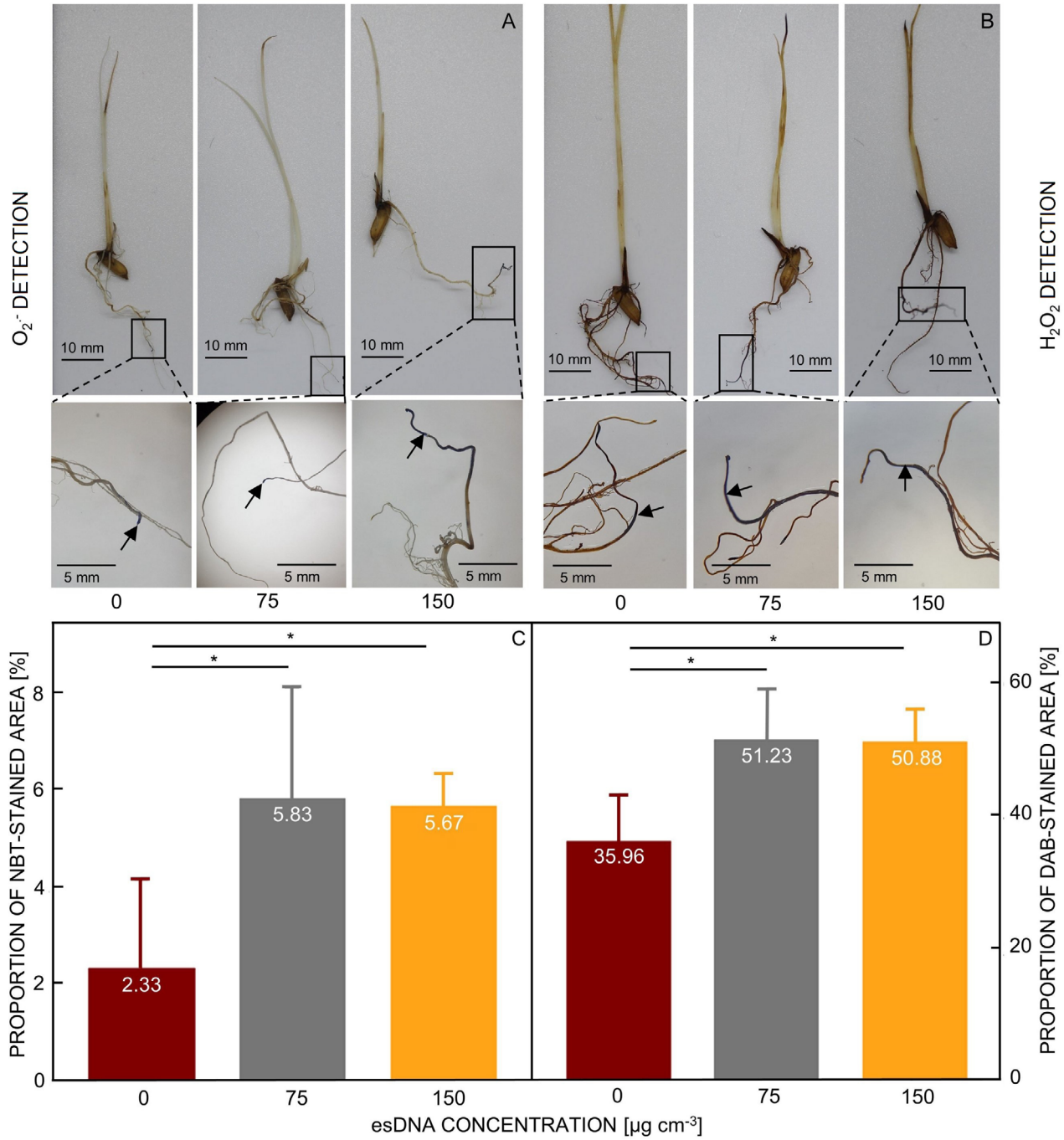

Fig. 3. Semi-quantitative determination of ROS production in esDNA-treated rice roots. Superoxide (O_2^-) was detected by NBT staining (*A*), while hydrogen peroxide (H_2O_2) by DAB staining (*B*). Stained areas were mostly detected closer to the tip of the roots which was blue and brown representing superoxide and hydrogen peroxide accumulation, respectively. The insets of the figure show the magnified area of stained root tips, which are also pointed by the *arrow*. Increased proportion of NBT- (*C*) and DAB- (*D*) stained areas were observed in roots treated with esDNA, but the increase was not correlated to esDNA concentration. The values were obtained from 10 treated rice roots. Statistically significant values (Duncan Multiple Range Test, $P < 0.01$) are indicated by *asterisks* above the bars.

as a DAMP (Ferrusquía-Jiménez *et al.* 2020), in which its presence is translated as a danger signal by the constant surveillance of the immune system (Matzinger 1994). However, to our best knowledge, there is no record of how monocot plants respond to exposure to esDNA in terms of growth inhibition. A study in *Z. mays* (Barbero *et al.* 2016) was focusing on the analysis of plasma membrane depolarization and Ca^{2+} signaling upon esDNA treatment. Another study on monocot (Le Mire *et al.* 2019), showed an elicited immune response in *T. aestivum* following the treatment of non-self exDNA from pathogenic fungi (*Zymoseptoria tritici*). The difference in early response to

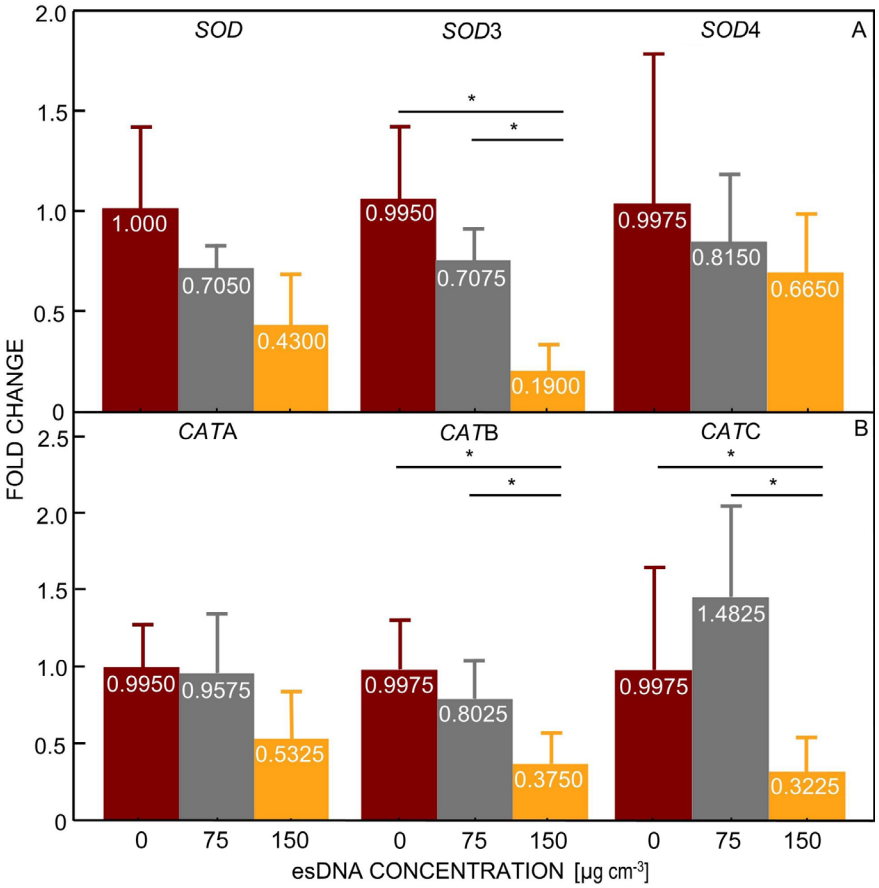

Fig. 4. Relative expression of genes encoding antioxidative enzymes superoxide dismutases (SODs) and catalases (CATs) in esDNA-treated rice roots on 7 dpt. Significantly lower expression of *SOD* (*A*) and *CAT* (*B*) was observed in *SOD*3 isoform and *CAT*B-C isoforms, respectively, in 150 µg cm^{-3} compared to 0 and 75 µg cm^{-3} esDNA-treated roots. Gene expressions of *SOD, SOD*4, and *CAT*A were not significantly different among all treatment groups. The values were obtained from 5 RNA extracts, which originated from a total of 100 rice roots. Statistically significant values (Duncan Multiple Range Test, $P < 0.01$) are indicated by *asterisks* above the bars.

esDNA treatment was recorded in *Z. mays* and *P. lunatus* (a C_4 monocot and a C_3 dicot, respectively), in which a much less concentration of esDNA was required to induce the plasma membrane potential depolarization and Ca^{2+} signaling in *P. lunatus* than in *Z. mays* (Barbero *et al.* 2021). The author argued that this might be due to the distinct metabolism between C_4 and C_3 plants and the dimorphic nature of *P. lunatus* mesophyll cells. Conducting this study in rice (*Oryza sativa* cv. MSP13), as the model plant for monocots, to determine the responses to esDNA treatment at the level of gene expression, physiology, and phenotype could potentially support the exploration of DNA-based biopesticide or biostimulant.

In our study, based on length measurements, inhibition of growth was observed in roots but not in the shoot of rice on 7 dpt. We also found that the fresh mass of esDNA-treated plants was not significantly lower compared to the non-treated ones. This concentration-dependent growth inhibition in rice root agrees with the other studies in dicot plants such as in *Acanthus mollis* (Mazzoleni *et al.* 2015) in *L. sativa* (Vega-Muñoz *et al.* 2018), in *P. vulgaris* (Duran-Flores and Heil 2018), and in *A. thaliana* (Chiusano *et al.* 2021). This indicates that although monocot required a higher concentration of esDNA to induce changes in membrane depolarization within 30 min upon contact (Barbero *et al.* 2016), the subsequent signaling processes might be similar that after a prolonged exposure eventually led to growth inhibition. Züst and Agrawal (2017) emphasized that upon sensing danger, plants often decide on a defense trade-off, enabling them to fine-tune their phenotype response even by constraining growth. This antagonism between defense and growth is genetically programmed but can be uncoupled by the rewiring of regulatory pathways (Guo *et al.* 2018).

The changes in architecture and number of lateral and crown roots in rice are involved in responses to stress *via* auxin signaling and gene expression modulation (Seo *et al.* 2020). Under drought conditions, lateral roots formation increased to multiply surface area for water absorption and maintain productivity (Kim *et al.* 2020). Crown root formation was also compromised in Zn-deficient rice as indicated by the delayed formation and decreased number (Nanda and Wissuwa 2016). Hence, our result suggests the response of lateral and crown roots in rice to esDNA exposure is distinct from responses to drought or Zn-deficiency. Unfortunately, literature on biotic stress in rice

that focuses on the number of crown and lateral roots is scarce.

Based on the limited growth of the root but not of the shoot, there is an indication that growth inhibition to esDNA exposure is a confined response only within the local organ (root) until at least 7 dpt. This is also supported by the measurement of the greenness index in all treatments which showed that there was no significant difference. The greenness index of the leaves indirectly measures leaves health, a high percentage of green indicates healthy leaves, while non-green areas such as brown or yellow might suggest chlorosis or infection. However, except for the chlorotic leaves in *A. thaliana* (Chiusano *et al.* 2021), none of the aforementioned studies in dicot determined shoot phenotypes. Compared to our study, Chiusano *et al.* (2021) exposed the plant to a higher concentration of fragmented esDNA within a more extended period. Long-distance signaling in plants is known to involve various mobile molecules, such as small proteins, peptides, RNAs, metabolites, and second messengers, transmitting extracellular stimuli from sensing tissues to target organs (Takahashi and Shinozaki 2019). For instance, rhizobial inoculation to *Lotus japonicus* roots upregulated the production of CLE-RS2 glycopeptide, which was then translocated to the shoot *via* xylem to elicit the subsequent signaling events that were eventually transmitted back to the root to avoid excessive nodule formation (Notaguchi and Okamoto 2015). It was recorded that a minimum concentration of CLE-RS2 glycopeptide to induce such signaling events was 100 nM (Okamoto *et al.* 2013). Once the minimum content is satisfied, long-distance signaling also requires ample time for a slower more specific signal to develop and move to distal parts of the plant (Choi *et al.* 2017). Hence, we suggest that the concentration and period of exposure to signaling/damage molecules, such as esDNA, determines whether the signal is translocated to the distant organs or is only responded to by the local ones.

The immediate growth inhibition was not found in plants treated with extracellular non-self DNA (nsDNA). Serrano-Jamaica *et al.* (2021) did not confirm a significantly shorter *Arabidopsis thaliana* until 22 dpt of direct spraying of 60 and 100 µg cm^{-3} DNA mixture of *Phytophthora capsici*, *Fusarium oxysporum*, and *Rhizoctonia solani* on the leaves. Similarly, Mazzoleni *et al.* (2015) and Vega-Muñoz *et al.* (2018) found that only the exposure to esDNA induced root growth inhibition in *A. mollis* or suppression of germination rate in *L. sativa*. High specificity, within the same genus, in determining response towards self *vs.* non-self DNA is demonstrated in *P. vulgaris* that root growth is inhibited by esDNA but not by nsDNA of *P. lunatus* (Duran-Flores and Heil 2018). Monocot weeds are especially troublesome since they cannot be selectively eliminated due to the similar molecular systems to that of crop plants. Hence, once the concentration and period of exposure are optimized to a combination for eliminating competition between monocot crops and weeds, this species-specific response hindering growth is potentially explored to develop DNA-based biopesticide. However, the exact receptors or mechanisms of how plants can distinguish between esDNA and nsDNA and further tailor their responses remain a matter of speculation.

There are at least four proposed modes of extracellular DNA recognition by plant cells; three modes involve the internalization of the DNA, while one mode only requires detection by plasma membrane receptor(s) to trigger a cascade of intracellular responses (Bhat and Ryu 2016). These responses involve ROS modulation following exposure to esDNA or nsDNA (Yakushiji *et al.* 2009), which are relayed to initiate phenotypic responses. Our results show that exposure to esDNA enhanced ROS accumulation in roots that is independent of concentration unlike growth inhibition, which is negatively correlated and concentration-dependent. Contrasting to Yakushiji *et al.* (2009), Duran-Flores and Heil (2018) correlated ROS regulation to the specificity of response: the exposure to esDNA of *P. vulgaris* increased H_2O_2 content but not the exposure to nsDNA. Regarding ROS signaling, this study confirms that the defense trade-off mechanism following damage sensing through esDNA exposure involves $O_2^{\cdot-}$ and H_2O_2 regulation. According to Mittler (2017), increasing ROS content correlates to a shift from cytostatic to redox biology (such as cell proliferation) and then to cytotoxic (oxidative stress) when it gets too high. This further raises the question of whether growth inhibition in the exposed area to esDNA was due to cell cycle arrest (cytostatic) or physiological cell death (cytotoxic). Although the increasing ROS content seems to point to physiological cell death, Chiusano *et al.* (2021) proposed ROS regulation is a part of signal propagation following esDNA exposure, which eventually initiates cell cycle arrest. Thus, to develop a DNA-based biopesticide, it is crucial to determine esDNA concentration that pushes the redox state of the weed cells to oxidative stress and further to cell death or cell cycle arrest.

We then measured the expressions of genes to correlate the activation of the enzymatic antioxidant system to the elevated production of $O_2^{\cdot-}$ and H_2O_2. Superoxide dismutases (SODs) are a group of enzymes that play a major role in combating oxygen radical-mediated toxicity (Sen Raychaudhuri and Deng 2000). The enzyme alternately catalyzes the dismutation of the superoxide radical into H_2O_2 and O_2. H_2O_2 is then scavenged by catalases (CATs), which are known for their function in plant defense, aging, and senescence (Yang and Poovaiah 2002). Our results show that only the expressions of *SOD*3, *CAT*B, and *CAT*C were regulated. This suggests that those three genes could be key genes in quenching ROS in rice roots when responding to esDNA exposure. Similarly, Vighi *et al.* (2017) found that *SOD* and *CAT* were regulated following salt stress and were only characterized by some isozymes. Modulation of ROS in plants upon receiving abiotic or biotic stresses is accounted to set the intracellular redox state to optimize signaling processes. This is then followed by the regulation of antioxidant systems so that the cellular redox state does not enter the cytotoxic level (Mittler 2017). Our results suggest that the downregulated expression of 3 antioxidant genes on 7 dpt has led to the tipped-off cellular redox state in roots to cytotoxicity that inhibited their growth.

For future studies, it is worth exploring the esDNA role

as a biostimulant for monocot crops. Duran-Flores and Heil (2018) showed that esDNA reduced infection by the bacterial pathogen in *A. mollis*. Similarly in *A. thaliana,* the exposure to esDNA induced resistance against pathogens *Botrytis cinerea, Hyaloperonospora arabidopsidis,* and *Pseudomonas syringae* and against aphid infestation (Rassizadeh *et al.* 2021). The genes expression in *Solanum lycopersicum* involved in plant-biotic interactions were also modulated by the esDNA treatment, such as the pathogenesis-related proteins (PRPs), calcium-dependent protein kinases (CPK1), heat shock transcription factors (Hsf), heat shock proteins (Hsp), receptor-like kinases (RLKs), and ethylene-responsive factors (ERFs) (Barbero *et al.* 2021). It is expected that the application of lower concentrations of esDNA to monocot plants could be explored as biostimulants, while higher doses as biopesticides.

Duran-Flores and Heil (2018) documented that the simple application of homogenates from self leaves activated defense responses and suggested that esDNA is one of the agents causing DAMP effect in plants. However, the use of crude leaves extract is thought to also expose several other molecules that do not induce DAMP-related responses or even suppress the biostimulants or bioherbicides effect by interfering with the sensing of esDNA or lower esDNA stability. Thus, the application of esDNA as bioherbicide or biostimulant on an agricultural scale requires careful planning. The use of removed weeds or waste material from the crops themselves is considered suitable as intact DNA from fresh tissues is not required. Furthermore, extraction of already fragmented DNA is beneficial to reduce the cost of sonication. Carbajal-Valenzuela *et al.* (2021) emphasized the need to optimize methods for industrial-scale DNA extraction, especially in the major processes of tissue lysis and centrifugation. The use of mechanical apparatus and methods at an industrial scale for cell lysis and centrifugation, to obtain comparable results to lab-scale extraction, are reviewed by Piccinno *et al.* (2016) and Shehadul Islam *et al.* (2017). For field application, the use of a stabilizing agent with antimicrobial properties, such as chitosan (Meitha *et al.* 2022), could be considered to prolong or even enhance the effects.

Conclusions

Extracellular self-DNA (esDNA) is now categorized as DAMP and known to trigger various responses that might be explored for developing species-specific bioherbicides or biostimulants. Our study confirms that in rice, a model for monocots, esDNA exposure inhibits growth in the local organ (root) but not in the distant one (shoot). Similar to the esDNA studies in dicots, the increased production of $O_2^{\cdot-}$ and H_2O_2 is also involved in the subsequent signaling steps. The response was characterized by the suppressed expression of antioxidant genes *SOD*3, *CAT*B, and *CAT*C. The local inhibition in root and healthy phenotypes of the shoot could indicate that a higher concentration and more extended period of esDNA exposure are required to induce cell death or cell cycle arrest. Thus, it is interesting for future research to determine the optimum concentration and period of exposure to esDNA that inhibit total growth of specific weed species but bring no or minimum effect to crop species. Furthermore, defining that esDNA is species-specific but that it does not regard individual variations in monocots could potentially be the solution to herbicide-resistant species.

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