# Gene Expression Related to Oxidative Stress Induced by Herbicides in Rice

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## ABSTRACT

Herbicides are stressors that can have negative effects on plants. In *Oryza sativa* (L.), differential gene expression may be evaluated through real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR). The aim of this study was to evaluate the stability of candidate reference genes and to quantify the relative expression of oxidative stress genes at different times (12, 24, 48, and 96 hours after treatment [HAT]) with penoxsulam, cyhalofop-butyl, and bentazon herbicides. Norm-Finder, BestKeeper, and GeNorm software and the comparative ΔCt method were used to assess expression stability and to determine the RT-qPCR threshold values of the candidate reference genes. The *UBQ5* gene was the most stable among the reference genes analyzed. The gene expression results showed upregulation of *OsCAT* and *OsMnSOD1* genes at all times after herbicide application. The *OsAPX2* and *OsGST3* genes showed increased gene expression at 12 and 96 HAT for all herbicides. The *OsHO-1* gene had the most significant expression changes, with maximum expression levels at 24 HAT with bentazon and at 96 HAT with penoxsulam and cyhalofop-butyl. Overall, antioxidant system gene expression increased after the application of bentazon, penoxsulam, and cyhalofop-butyl in rice.

## Core Ideas

- *UBQ5* was the most stable reference gene for gene expression analysis in rice treated with herbicides.
- The herbicides increased gene expression associated with defense against oxidative stress.
- *OsHO-1* showed the greatest change in expression in response to herbicide treatment.

**P**LANT RESPONSES to abiotic and biotic stresses are regulated by gene expression regulating different physiological processes, enabling survival in different environmental conditions. In many crops, herbicides are considered an important source of abiotic stress because they inhibit enzymes, proteins, and other key points of primary and secondary metabolism, leading to oxidative stress (de Freitas-Silva et al., 2017). However, in addition to having a profound impact on weed control in agriculture, herbicides have played a major role in expanding the understanding of fundamental plant processes through their use as molecular probes (Dayan et al., 2010).

In susceptible plants, herbicides can have a variety of effects. Bentazon [3-(1-methylethyl)-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide] inhibits photosynthesis by competing with the target site of plastoquinone B in $D_1$ protein of photosystem II. Penoxsulam [2-(2,2-difluoroethoxy)-*N*-(5,8-dimethoxy[1,2,4]triazolo[1,5-*c*]pyrimidin-2-yl)-6-(trifluoromethyl)benzene-sulfonamide] affects the acetolactate synthase enzyme and the production of amino acids such as valine, isoleucine, and leucine. Cyhalofop-butyl [(2*R*)-2[4-(4-cyano-2-fluorophenoxy)phenoxypropanoate] is an inhibitor of acetyl CoA carboxylase, which controls the first step in fatty acid synthesis (Senseman, 2007). In southern Brazil, bentazon, penoxsulam, and cyhalofop-butyl herbicides are commonly sprayed on irrigated rice for weed control. Injury to the crop can occur due to the modes of action of these herbicides, which result in metabolic degradation of glutathione-S-transferase, cytochrome $P_{450}$ monooxygenase, and glycosyltransferases, and due to increased antioxidant enzyme activity (Délye et al., 2013).

Although these herbicides are recommended for use on irrigated rice, they may cause injury to plants by increasing reactive oxygen species (ROS) and other compounds with a high capacity for causing cell damage. At the cellular level, different ROS can be involved in metabolic regulation and physiological processes

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**Abbreviations:** APX, ascorbate peroxidase; CAT, catalase; Ct, threshold cycle; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; HAT, hours after treatment; RE, relative expression; ROS, reactive oxygen species; RT-qPCR, real-time quantitative polymerase chain reaction; SOD, superoxide dismutase; UBC-E2, ubiquitin conjugating enzyme E2.

Table 1. Relation of candidate reference genes and primers used for the RT-qPCR in rice in response to herbicide exposure.

| Gene | Forward primer (5′–3′) | Reverse primer (5′–3′) | Efficiency | Reference |
|---|---|---|---|---|
| *ACT11* | CAGCCACACTGTCCCCATCTA | AGCAAGGTCGAGACGAAGGA | 2.00 | Zhang and Hu, 2007 |
| *β-Tubulina* | GCTGACCACACCTAGCTTTGG | AGGGAACCTTAGGCAGCATGT | 1.64 | Zhang and Hu, 2007 |
| *Eef-1a* | TTTCACTCTTGGTGTGAAGCAGAT | GACTTCCTTCACGATTTCATCGTAA | 1.72 | Zhang and Hu, 2007 |
| *UBC-E2* | CCGTTTGTAGAGCCATAATTGCA | AGGTTGCCTGAGTCACAGTTAAGTG | 1.84 | Jain et al., 2006 |
| *eIF-4-a* | TTGTGCTGGATGAAGCTGATG | GGAAGGAGCTGGAAGATATCATAGA | 1.84 | Jain et al., 2006 |
| *UBQ10* | TGGTCAGTAATCAGCCAGTTTGG | GCACCACAAATACTTGACGAACAG | 2.55 | Jain et al., 2006 |
| *UBQ5* | ACCACTTCGACCGCCACTACT | ACGCCTAAGCCTGCTGGTT | 2.00 | Jain et al., 2006 |
| *GAPDH* | AAGCCAGCATCCTATGATCAGATT | CGTAACCCAGAATACCCTTGAGTTT | 2.14 | Jain et al., 2006 |
| *TIP41* | GTTTGGATGAACCCCGCAA | GGCAACAAGGTCAATCCGATC | 1.75 | Caldana et al., 2007 |
| *Cyclophilin* | CCACCATCACAGATCGGATCTT | GCGGTCAGAGCGAAAGTAGCTA | 1.80 | Caldana et al., 2007 |
| *18S rRNA* | CTACGTCCCTGCCCTTTGTACA | ACACTTCACCGGACCATTCAA | 2.00 | Jain et al., 2006 |
| *OsHO1* | GGAGGAGAAGGACCATTG | GAATATGTGACGGAGGAGAT | 1.98 | Zhang et al., 2014 |
| *OsAPX2* | AGAGTCAGTACGATCAAGAC | TCTTGACAGCAAATAGCTTGG | 2.09 | Zhang et al., 2014 |
| *OsGST3* | CAAGATGAAGCAGGCAGAG | GCACACCAACACCAACTT | 2.18 | Zhang et al., 2014 |
| *OsCAT* | GTCCATGCTTTCAAGCCAAGTC | TCCATGTGCCTGTAGTTGAGTG | 2.08 | GenBank: DQ078758.1 |
| *OsMnSOD1* | CTACGTCGCCAACTACAACA | CTGATAGGCTTGAGGTTATTCCAG | 1.92 | GenBank: L34038.1 |

of plants under stress by activating antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), peroxidases, and glutathione-S-transferases (Dixon et al., 2010; Mittler, 2017). In plants, the induction of oxidative stress can occur in different cellular compartments due to the activation of distinct signal transduction pathways that promote physiological responses and regulate normal cellular processes (Mittler, 2017; Shekhawat and Verma, 2010). Additionally, previous studies have reported differential gene expression as a mechanism of cellular defense in plants undergoing herbicide treatment (Fang et al., 2015; Liang et al., 2012).

Real-time quantitative polymerase chain reaction (RT-qPCR) is a powerful approach to evaluate gene expression as a response to herbicides. However, this technique requires the identification and validation of stably expressed genes for normalizing the results. These internal control genes are used to normalize RT-PCR expression analysis by minimizing the differences caused by sampling techniques, RNA quality and quantity, the presence of inhibitors in certain tissues, and variations in the reverse transcription reaction (Dombrowski and Martin, 2009). Several reports describing the proper selection and evaluation of multiple reference genes as internal control genes for accurate normalization of RT-qPCR have been published (Bail et al., 2008; Vandesompele et al., 2002). The aim of this study was to evaluate the stability of candidate reference genes for transcript-level normalization in herbicide-treated irrigated rice and to evaluate antioxidant gene expression after the application of penoxsulam, cyhalofop-butyl, and bentazon herbicides.

## MATERIALS AND METHODS
### Plant Material and Experimental Design

The experiment was performed in a greenhouse using a completely randomized design with three replications. Sixteen seedlings of the cultivar IRGA 424 (tolerant to penoxsulam, cyhalofop-butyl, and bentazon herbicides) were planted in plastic pots with a volumetric capacity of 8 L in soil classified as Yellow Red Argisol and managed in accordance with the technical recommendations for the crop. The herbicide treatments were penoxsulam (Ricer, 240 g a.i. L$^{-1}$, SC; Dow Chemical

Co., Brazil) at a rate of 60 g ai ha$^{-1}$, cyhalofop-butyl (Clincher, 180 g a.i. L$^{-1}$, EC; Dow Chemical Company-Brazil) at a rate of 315 g ai. ha$^{-1}$, bentazon (Basagran, 480 g ai. L$^{-1}$, SL; BASF Chemical Co., Brazil) at a rate of 960 g ai ha$^{-1}$, and the control (no herbicide application). Herbicide spray was applied when plants had three to four true leaves (~15 d after emergence) using a $CO_2$ backpack sprayer with Teejet TT110.02 nozzles at a spraying volume of 150 L ha$^{-1}$. Flood irrigation occurred 1 d after herbicide application, and rice leaf samples (bulk of 16 seedlings) were collected at 12, 24, 48, and 96 hours after treatment (HAT). Samples were stored at –80°C.

### RNA Extraction and cDNA Preparation

Total RNA was extracted from the rice leaves using PureLink Plant RNA Reagent (Invitrogen) and digested with DNase I (Invitrogen), according to the manufacturer's instructions, to degrade DNA contaminants. The integrity of the extracted RNA was determined by 2% agarose gel electrophoresis (p/v), and RNA quantity and quality were measured by 1% agarose gel electrophoresis (p/v) and with a Nanophotometer (NanoDrop 2000, Thermo Scientific) with absorbance ratios of 260/280 and 260/230 nm; values close to 2 were considered acceptable for use in RT-qPCR. The cDNA was obtained using a SuperScript First-Strand System Kit for RT-qPCR (Invitrogen) as recommended by the manufacturer.

### Candidate Reference Genes and Conditions for RT-qPCR

Candidate reference genes for rice that were considered possibly stable for normalization were evaluated, and 11 primer pairs reported in previous studies were used as internal controls in the RT-qPCR analyses (Table 1). The gene sequences of catalase (*OsCAT*) and superoxide dismutase (*OsMnSOD1*) were obtained from rice transcripts deposited in the National Center for Biotechnology Information, and primers were designed using Primer 3 software (Untergasser et al., 2012). For the ascorbate peroxidase (*OsAPX2*), glutathione S-transferase 3 (*OsGST3*), and heme oxygenase (*OsHO-1*) genes, primers were obtained from previous studies that described the selection of reference genes (Table 1).

For the amplification reaction, 6.25 µL of LightCycler 480 SYBR Green I Master Mix (Roche Applied Science), 0.5 µM of primer (10 mM), 1 µL cDNA (0.2 mg), and water (to complete the final volume of 12 µL) were used. Using a LightCycler 480 system (Roche Applied Science), the amplification conditions were as follows: one cycle at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 20 s, 60°C for 15 s, and 72°C for 20 s. This process was terminated by a dissociation curve at 95°C for 5 s, then incubation at 70°C for 1 min, followed by gradual heating in 0.11°C steps until 95°C was reached. The product was finally incubated at 40°C for 30 s, and all reactions were performed in three replicates for each cDNA sample. No-template controls were included, and the purity of the amplicons was confirmed by the presence of a single peak in the melting curve.

### Efficiency and Analysis of Reference Gene Expression Stability

Polymerase efficiency was measured using four serial dilutions of the cDNA (1:1, 1:5, 1:25, and 1:125) to generate a standard curve for each primer pair tested. The E value was estimated by the equation $E = 10^{(-1/slope)}$ (Rasmussen, 2001), with values between 1.8 and 2.2 considered acceptable for the reference genes. The ranking and performance of the reference genes were assessed by determining the mean threshold cycle (Ct) values in each sample (1:25 dilution) of the RT-qPCR. Changes in rice gene expression were evaluated with the RefFinder tool and were integrated with the algorithms in GeNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004) software and by the ΔCt comparative method, which is widely used to estimate expression stability and to select the appropriate candidate target genes (Chen et al., 2011). The average Ct value of each sample was used as input data on the website.

Target gene results were compared with gene expression changes in rice for each treatment (herbicides and sampling time) in reference to the control (without herbicide). The relative level of expression (Q) for a given gene was calculated based on the formula $Q = 2^{(-\Delta\Delta Ct)}$ (Livak and Schmittgen, 2001), where the $\Delta\Delta Ct = Ct_{samples} - Ct_{min}$ ($Ct_{min}$ is the sample with the lowest Ct value among all samples, and $Ct_{samples}$ represents each sample by Ct value). The results were assessed by ANOVA, and the means were compared by Tukey's test ($p \leq 0.05$).

### RESULTS
### Analysis of Reference Gene Expression Efficiency and Stability

The determination of a reference gene to study relative expression (RE) is the first step in analyzing a target gene. The amplification efficiency of the reference primers was calculated individually by logarithm (Log) cDNA dilutions. Our results demonstrate that the most adequate dilution was 1:25, which was then used to validate the target genes. Among the genes tested, the efficiencies ranged from 1.64 for *β-tubulin* to 2.55 for Ubiquitin 10 (*UBQ10*). 18S ribosomal RNA (*18S*), eukaryotic initiation factor 4A (*elF-4a*), *cyclophilin*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ubiquitin conjugating enzyme E2 (*UBC-E2*), ubiquitin 5 (*UBQ5*), and *β-tubulin* showed efficiency values ranging from 1.80 to 2.14 and were thus considered adequate reference genes. However, eukaryotic elongation factor

Table 2. General mean, SD, and CV% of seven endogenous genes in rice.

| Gene | General mean | SD | CV% |
|---|---|---|---|
| *ACT11* | 31.03 | 1.49 | 4.81 |
| *UBC-E2* | 30.83 | 1.26 | 4.09 |
| *elF-4-a* | 35.70 | 1.38 | 3.86 |
| *UBQ5* | 28.16 | 0.91 | 3.25 |
| *GAPDH* | 28.69 | 2.30 | 8.02 |
| *Cyclophilin* | 28.95 | 1.47 | 5.10 |
| *18S rRNA* | 19.77 | 2.52 | 12.75 |

1-α (*Eef-1α*), *UBQ10*, and aquaporin (*TIP41*) did not show adequate values (Table 1) and were excluded from further analysis.

The specificity of the rice reference genes was calculated by average Ct value, SD, and CV; *UBQ5* was the best reference for use in RT-qPCR, with an average value of 28.16, a low SD (0.91), and low CV (3.25%) (Table 2). Accuracy of the analysis was evaluated by M values using NormFinder, BestKeeper, and GeNorm software as well as the ΔCt comparative method. The most stable reference genes in rice under herbicide stress, as determined by NormFinder software, were *UBQ5* and *UBC-E2*, with M values of 0.76 and 1.2, respectively (Fig. 1A). *18S* and *GAPDH* showed higher M values of 2.18 and 2.85, respectively (Fig. 1B).

Evaluating the same datasets with the BestKeeper software, which is used to estimate stability by SD with a cutoff value of 1, indicated that only *UBQ5* was stable (SD = 0.93) compared with all other reference genes (SD > 1) (Fig. 1C). Similar results were obtained by the ΔCt comparative method, where *UBQ5* was the most stable (M = 2.01), whereas *GAPDH* was the least stable (M = 2.85) (Fig. 1D). Additionally, this method of evaluation showed an expression stability ranking equal to that of NormFinder and similar to that of BestKeeper. Based on GeNorm software, the stability of the reference genes was evaluated by M value, with cutoff values of <1.5 (M < 1.5) considered acceptable; *elF4-a* and *actin* were the most stable (M = 1.47). *GAPDH* was the least stable (M = 2.42) for studying herbicide stress in rice (Fig. 1E).

### Oxidative Gene Expression in Response to Herbicides after Different Sampling Times

Bentazon, penoxsulam, and cyhalofop-butyl induced the expression of *OsCAT*, *OsMnSOD1*, *OsAPX2*, *OsGST3*, and *OsHO1* in rice plants after herbicide application. All genes analyzed, except *OsAPX2*, showed significant differences in expression after herbicide spraying and at different sampling times in rice plants (Fig. 2 and 3). The results demonstrated upregulation of the *OsCAT* transcript in herbicide-treated rice compared with the control at all sampling times, except at 48 HAT for bentazon, where there was no increase in gene expression. Exposure to bentazon at 24 HAT (RE = 3.43) and cyhalofop-butyl at 96 HAT (RE = 3.97) caused upregulation compared with the control, differing statistically from the other herbicides and sampling times (Fig. 2A).

For *OsMnSOD1*, greater upregulation occurred at 24 and 96 HAT in rice plants exposed to bentazon and cyhalofop-butyl (RE = 6.29 and 9.33, respectively). A lower level of transcript expression (RE = 1.03) was found with bentazon application at 96 HAT, which was not different from that of the control (Fig. 2B).

Upregulation of *OsGST3* with maximum expression at 96 HAT with cyhalofop-butyl (RE = 6.02) and bentazon (RE =
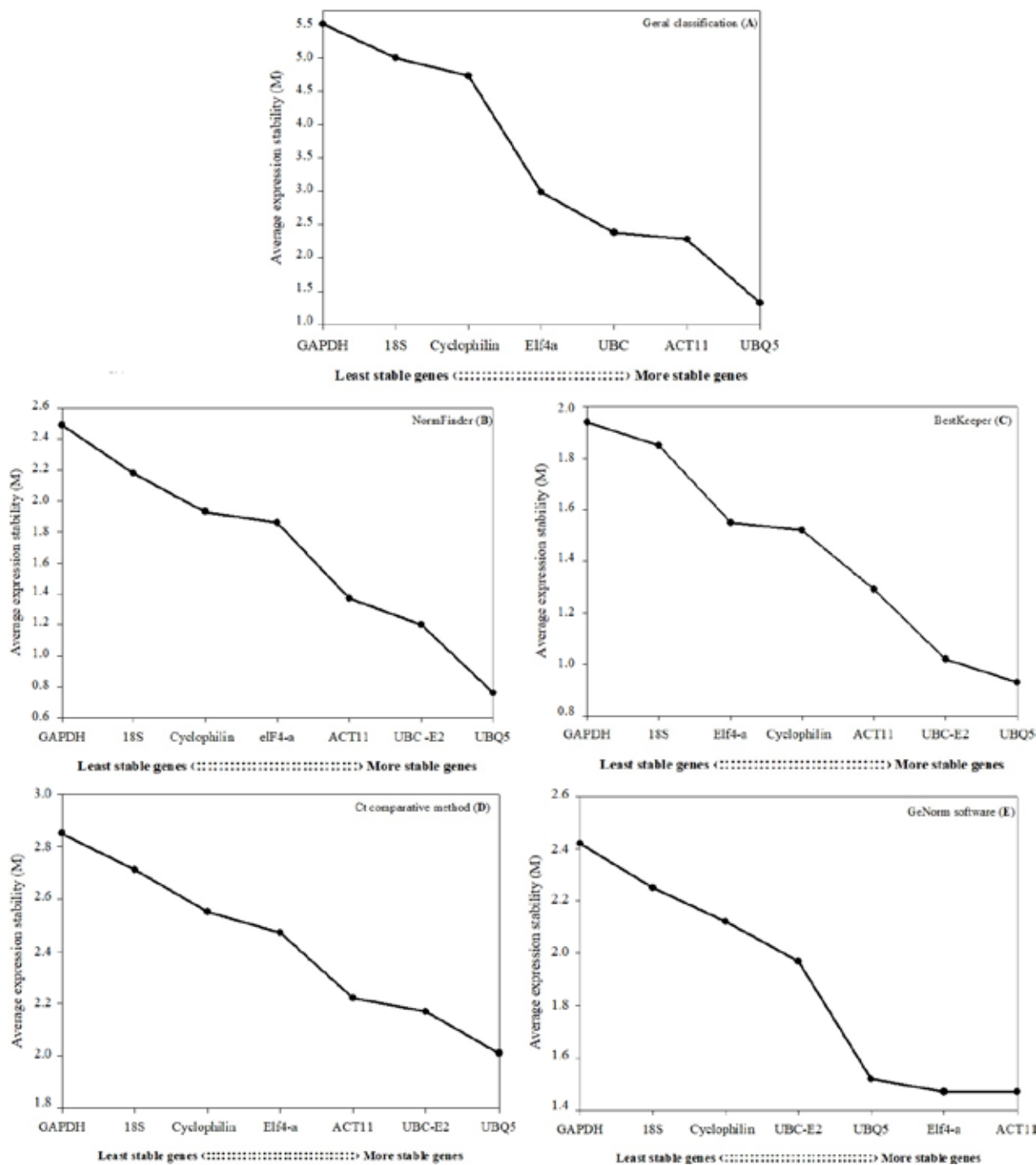
Fig. 1. General ranking (A) and average stability of expression (M value) according to NormFinder (B), BestKeeper (C), ΔCt comparative method (D), and GeNorm software (E) of the seven reference genes to rice after application of penoxsulam, cyhalofop-butyl, and bentazon.

3.62) differed from the control (Fig. 2C). For penoxsulam, the RE of *OsGST3* showed the maximum level at 96 HAT with a value of 1.64, which differed only in comparison to 48 HAT (RE = 0.25). In addition, the results showed downregulation of this gene in rice plants for every herbicide evaluated at 48 HAT (Fig. 2C). For *OsHO1*, the application of penoxsulam caused an increase in gene expression regardless of the time at evaluation, with a maximum RE value of 12.34 at 96 HAT, differing from the other treatments at the same time evaluated. Moreover, more significant upregulation of this gene was observed with cyhalofop-butyl and bentazon herbicides at 24 HAT (RE = 11.04 and 9.14, respectively) (Fig. 2D). When *OsAPX2* was evaluated, an interaction between the herbicides and sampling times was not observed; although there

was a gradual increase at 12 HAT (RE = 1.56) and decreases at 24, 48, and 96 HAT (RE < 1) (Fig. 3).

## DISCUSSION
### Efficiency and Stability of Candidate Reference Genes

Reliable quantification by RT-qPCR is based on the stable expression of reference genes over time and under different experimental conditions (Vandesompele et al., 2002), which is considered an important step in comparing gene expression and enabling the greatest RT-qPCR efficiency (Bustin et al., 2009). This study is the first to be performed on a set of candidate genes from irrigated rice for use in RT-qPCR with various algorithms
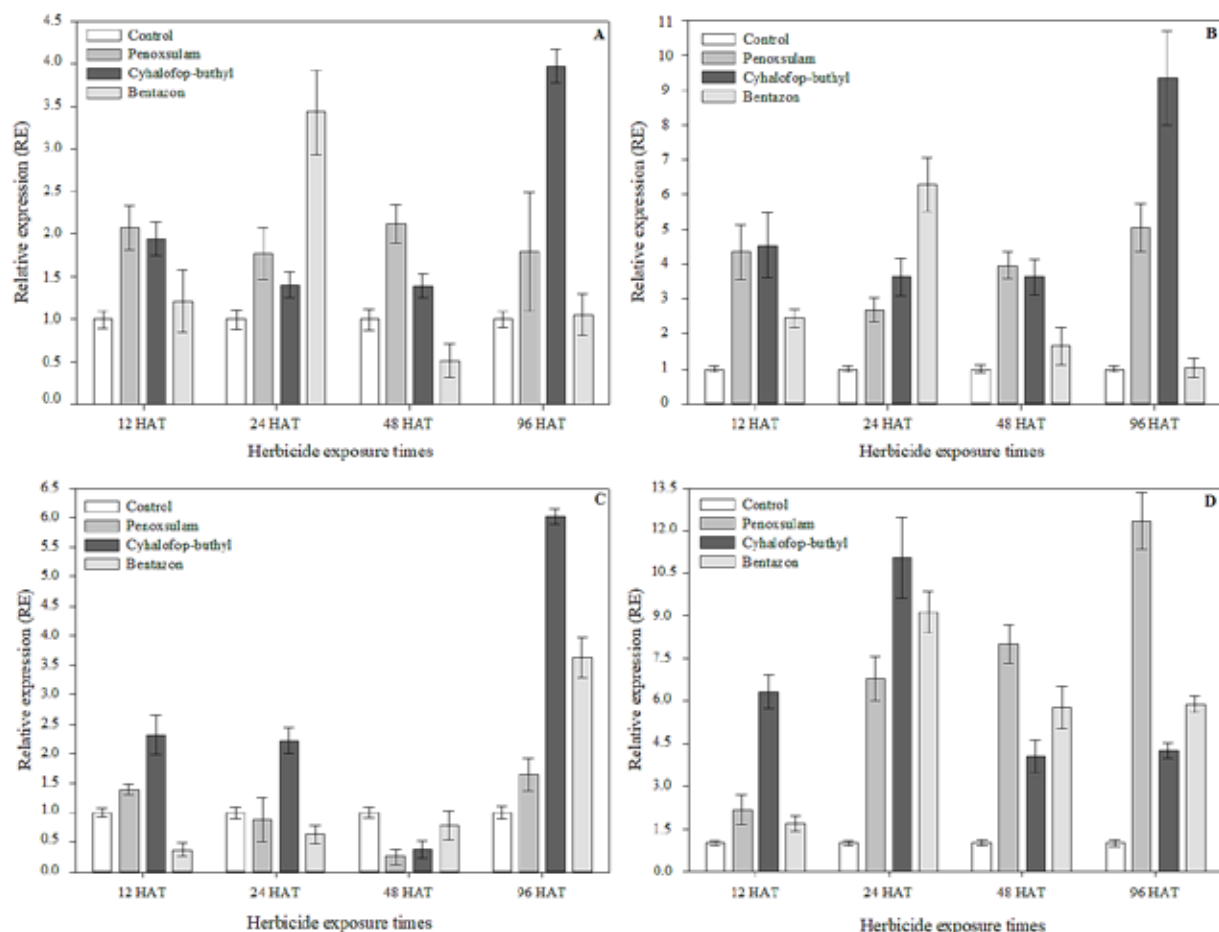
Fig. 2. Relative expression of *OsCAT* (A), *OsMnSOD1* (B), *OsAGST3* (C), and *OsAHO1* (D) in rice after application of penoxsulam, cyhalofop-butyl, and bentazon at different times. Vertical bars represent SEM.

(GeNorm, NormFinder, BestKeeper, and ΔCt comparative method), testing several herbicides and times after application. The best and most stable reference gene was *UBQ5*, whereas *GAPDH* was the least stable. Ubiquitins are highly conserved proteins among eukaryotic organisms; they carry out their functions through covalent attachment to other cellular proteins and are involved in numerous signaling processes (Sun et al., 1997), chromatin structure and transcription, DNA repair, endocytosis regulation, and protein trafficking (Hernandez-Garcia et al., 2009). *UBQ5* is also considered the most stable gene in *Arabidopsis* (Koch et al., 2012), *Oryza sativa* (Jain et al., 2006), and *Populus* (Brunner et al., 2004). Other studies have reported the use of different reference candidate genes in analyzing gene expression; the quality of the data from these analyses is affected by and dependent on the species and the environmental stress conditions under consideration (Iskandar et al., 2004). In soybean, it was found that *EF1A* and *ACT11* were the best reference genes for studying salinity stress, whereas *TUB4*, *TUA5*, and *EF1A* were the best for drought stress (Ma et al., 2013). For rice, 18S and 25S rRNA exhibited the most stable expression in plants grown under various environmental conditions (Jain et al., 2006). The need for validation of the most stable gene for a given experimental condition is important to highlight because there is currently no universal reference gene for use with different plant species, tissues, and stressors.

Herbicides are stressors that can cause injury and physiological disturbances that can induce an increase in ROS in plants (Maroli

et al., 2015). Oxidative stress is one response of plants exposed to herbicides; this response is associated with herbicide absorption, translocation, metabolization, and tolerance, and this stress can either lead to death or manifest quickly and transiently (Apel and Hirt, 2004). Excess ROS in plant cells causes changes in metabolism and antioxidant system activation and an increase in detoxification metabolites (Halliwell, 2006). Reactive oxygen species are reduced or excited states of atmospheric oxygen from aerobic metabolism necessary in several cellular processes, and excess ROS can cause cellular damage and death in plants (Sies et al., 2017).

An increase in the expression of antioxidant enzymes (APX, SOD, and CAT) after herbicide treatment is considered to be a plant defense response, and this response can change over time as a function of the mode of action of the herbicide and enhanced plant metabolism (Asada, 1999). Here, *OsCAT* and *OsMnSOD1* showed similar responses to the different herbicides at the times evaluated. In both genes, upregulation was demonstrated in all treatments, whereas *OsCAT* was downregulated only in bentazon-treated plants at 48 HAT. *OsCAT* and *OsMnSOD1* showed greater expression at 24 and 96 HAT for bentazon and cyhalofop-butyl, respectively. In addition, when penoxsulam was applied, the *OsCAT* gene showed greater expression at 48 HAT and *OsMnSOD1* at 96 HAT. The first responses of rice plants to bentazon can be related to photosynthesis inhibition through competition for the quinone B binding site on the D1 protein of photosystem II, which causes thylakoid membranes to be exposed to exogenous oxygen ($^1O_2$)
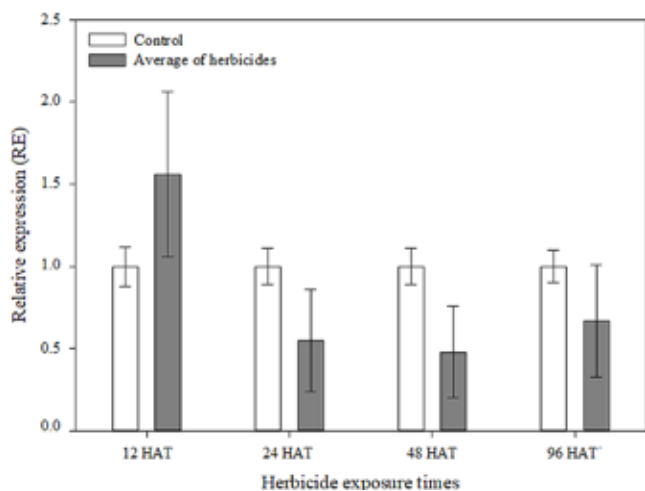
Fig. 3. Relative expression of *OsAPX2* gene in rice after application to penoxsulam, cyhalofop-butyl, and bentazon at different times. Vertical bars represent SEM.

and specific cleavage of the D1 protein (Okada et al., 1996). However, other ROS, such as $O_2$ and $H_2O_2$, can induce specific cleavage of the D1 protein in vitro. Similar reports showed upregulation of SOD in soybean leaves after the application of atrazine and bentazon, whereas downregulation of seven *OsAPX2* genes was observed after the use of glyphosate (Miyao et al., 1995). The present results demonstrate upregulation of *OsAPX2* at 12 HAT with bentazon, which did not differ from the other exposure times regardless of the herbicide used. The ascorbate peroxidase enzyme plays a key role in the conversion of $H_2O_2$ to water at different subcellular sites. The reasons for the reduced transcription of APX are not clear; one possibility is that catalases are located in the peroxisomes, whereas *OsAPX2* is cytosolic, resulting in differential expression levels. Increased expression of peroxisomal APX was similarly found in plants treated with atrazine and bentazon (Miyao et al., 1995).

Changes in ROS balance can produce adverse effects in plants due to the function of these molecules as secondary messengers and in diverse cellular signaling processes (Zhu et al., 2009). Herbicide stress plays a key role in the imbalance between endogenous and exogenous ROS, which can reduce antioxidant defenses or cause oxidative damage to cell components (Maroli et al., 2015; Zhang et al., 2009). Furthermore, plant antioxidant systems are important as a defense mechanism for cell protection and herbicide detoxification due to the activation of various enzymes involved in oxidation, reduction, hydrolysis, and conjugation reactions with sugar, amino acid, or glutathione, allowing increased solubility and reduced activity at the target site (Yuan et al., 2007).

In this study, the results demonstrated the upregulation of *OsAGST3*, *OsAHO1*, and *OsCAT* transcripts at 96 HAT with cyhalofop-butyl and *OsMnSOD1* with bentazon herbicides in relation to the control. In susceptible plants, photosystem II inhibitors led to rapid changes in photosynthesis compared with acetyl CoA carboxylase and acetolactate synthase inhibitors, which indirectly affect photosynthesis by causing lipid peroxidation and oxidative stress (Dayan and Zaccaro, 2012; Fang et al., 2015). The late expression of *OsGST3* in rice after the application of bentazon can change with phenological stage and occurs due to differential expression of the CYP81A6 gene and conjugation with glucose by insertion of radical OH into the aromatic ring (Lu et al., 2015). Moreover, GSTs are a set of enzymes that catalyze the conjugation of the reduced form of glutathione and are important for the regulation of different types of stress and for herbicide detoxification in plants (McGonigle et al., 2000; Soranzo et al., 2004). Differential expression of multiple GSTs was found in bentazon-treated soybean, with an increase in transcription at 4 HAT and a decrease at 8 HAT (Zhu et al., 2009). Increased expression of two GSTs has also been reported in rice plants after atrazine application (Zhang et al., 2014).

The expression of *HO-1* in plants has recently been the focus of research due to its key role in biosynthetic pathways, including biosynthesis of the phytochrome chromophore (Gisk et al., 2010), protection against oxidative damage (Shen et al., 2011), and root development (Guo et al., 2009). Its expression levels can be regulated by UVB radiation, $H_2O_2$, nitric oxide, cytokinin, and heavy metals (Xu et al., 2012). This study demonstrated differential upregulation of *OsHO-1* in rice after application of cyhalofop-butyl at 12 and 24 HAT and after application of penoxsulam at 48 and 96 HAT; these results are in contrast to the effects on the defense antioxidant system, in which downregulation of the *GST*, *SOD*, *CAT*, and *APX* genes occurred. Similarly, overexpression of *OsHO-1* was found in plants treated with atrazine and other herbicides (Liang et al., 2012; Zhang et al., 2009). *HO-1* is an inducible form of heme oxygenase that acts on oxidative signaling, and it is considered a dynamic indicator of oxidative stress tolerance and maintains cellular homeostasis (Cui et al., 2012).

Thus, *UBQ5* was found to be the most stable gene for normalization of a set of reference genes validated in irrigated rice for studies of herbicide stress using RT-qPCR. The herbicides penoxsulam, cyhalofop-butyl, and bentazon cause changes in the expression levels of *OsCAT*, *OsMnSOD1*, *OsAPX2*, *OsGST3*, and *OsHO-1*, which regulate the oxidative stress response in rice plants during response to injury and tolerance to herbicides. The *OsHO-1* oxidative stress gene can act as a positive regulator in response to penoxsulam, cyhalofop-butyl, and bentazon herbicides in irrigated rice.

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