



## Article

BENEMANN, D.P.<sup>1\*</sup>  
NOHATO, A.M.<sup>1</sup>  
VARGAS, L.<sup>2</sup>  
AVILA, L.A.<sup>1</sup>  
AGOSTINETTO, D.<sup>1</sup>

## IDENTIFICATION AND VALIDATION OF REFERENCE GENES FOR THE NORMALIZATION IN REAL-TIME RT-qPCR ON RICE AND RED RICE IN COMPETITION, UNDER DIFFERENT NITROGEN DOSES

*Identificação e Validação de Genes de Referência para a Normalização no Real-Time RT-qPCR em Arroz e Arroz Vermelho em Competição, sob Diferentes Doses de Nitrogênio*

**ABSTRACT** - Real time reverse transcription polymerase chain reaction (RT-qPCR) is an important technique to analyze differences in gene expression due to its sensitivity, accuracy, and specificity. However, before analyzing the expression of the target gene, it is necessary to identify and evaluate the stability of candidate reference genes for the proper normalization. This study aimed at evaluating the stability of candidate reference genes in order to identify the most appropriate genes for the normalization of the transcription in rice and red rice in competition under different nitrogen levels, as well as to demonstrate the effectiveness of the reference gene selected for the expression of the cytosolic ascorbate peroxidase (*OsAPX2*). Eleven candidate reference genes were assessed using the RefFinder which integrates the four leading software: geNorm, NormFinder, Bestkeeper, and the comparative delta-Ct method in addition to the analysis of variance to identify genes with lower standard deviation and coefficient of variation values. Eight out of the eleven genes have shown the desired effectiveness and, among them, the gene *UBC-E2* has the highest stability according to RefFinder and the analysis of variance. The expression of the gene *OsAPX2* has proven to be effective in validating the candidate reference gene. This study is the first survey on the stability of candidate reference genes in rice and red rice in competition, providing information to obtain more accurate results in RT-qPCR.

**Keywords:** *Oryza sativa*, gene expression, normalization.

**RESUMO** - Reação em cadeia da polimerase da transcrição reversa em tempo real (RT qPCR) é uma técnica importante para analisar a expressão diferencial dos genes, devido à sua sensibilidade, exatidão e especificidade. No entanto, antes da análise da expressão do gene alvo, é necessário identificar e avaliar a estabilidade dos genes candidatos a referência para a normalização apropriada. O objetivo deste trabalho foi avaliar a estabilidade de genes candidatos a genes de referência, a fim de identificar os genes mais adequados para normalização da transcrição em arroz e arroz-vermelho em competição, sob diferentes doses de nitrogênio, e demonstrar a eficácia do gene de referência selecionado pela expressão da ascorbato peroxidase citosólica (*OsAPX2*). Onze genes candidatos a referência foram avaliados usando o aplicativo online RefFinder, que integra os quatro principais programas de software: geNorm, NormFinder, BestKeeper e método comparativo delta Ct, bem como fez-se análise de variância para identificar genes

\* Corresponding author:  
<daiane\_bio@yahoo.com.br>

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<sup>1</sup> Universidade Federal de Pelotas, Pelotas-RS, Brazil; <sup>2</sup> Embrapa Wheat, Passo Fundo-RS, Brazil.

com menores valores de desvio-padrão e coeficiente de variação. Dos 11 genes, oito apresentaram eficiência dentro do padrão desejado, e, destes, o gene *UBC-E2* foi o mais estável. A expressão do gene *OsAPX2* mostrou-se eficiente para validação do gene candidato a referência. Este estudo é o primeiro levantamento sobre a estabilidade de genes de referência em arroz e arroz-vermelho em competição, fornecendo informações para obtenção de resultados mais precisos em RT-qPCR.

**Palavras-chave:** *Oryza sativa*, expressão gênica, normalização.

## INTRODUCTION

The analysis of gene expression is essential to understand several aspects of plant biology (Martin et al., 2008). The real time reverse polymerase chain reaction is currently one of the most powerful and sensitive techniques for the analysis of gene expression and it contributes to substantially improve the understanding of the signaling, development of metabolic pathways and cell processes (Paolacci et al., 2009). The reliable quantification through the analysis of real-time RT-qPCR of gene expression levels requires the normalization and control of many parameters, such as: initial sample size, RNA integrity, enzymatic efficiency of cDNA synthesis and PCR amplification and the transcriptional activity of tissue and cells analyzed (Bustin, 2002; Ginzinger, 2002). For the normalization, the use of internal control genes (reference genes) is the most reliable and convenient method to estimate the amount of initial RNA (Thellin et al., 1999), as well as to reduce possible errors made in quantifying the expression of the gene, which is obtained by comparing the expression levels in samples of the gene of interest and the genes constituting stable control (Paolacci et al., 2009).

It is likely that one or more genes are expressed constitutively in a specific organ an environment (Andersen et al., 2004). Thus, the systematic selection and validation of reference genes should be performed prior to all real-time RT-qPCR analyses (Gutierrez et al., 2008). Some genes are usually designated as reference genes due to their roles in basic cell processes, primary metabolism, and maintenance of cell structure (Czechowski et al., 2005; Wong and Medrano, 2005).

So, reference genes traditionally used in real-time RT-qPCR studies on plants include actine (Maroufi et al., 2010), tubulin (Wan et al., 2010), ubiquitin (Chen et al., 2011), 18S ribosomal RNA (Jain et al., 2006), and 40S ribosomal RNA (Cruz-Rus et al., 2011). Therefore, the reliability of the results of gene expression depends on the use of reference genes suitable for culturing and study conditions. To date, studies on reference genes in weeds are scarce and only a few reference genes have been validated under herbicide stress in a study regarding *Alopecurus myosuroides* and herbicides inhibitors of acetyl-CoA (Petit et al., 2012) and acetolactate-synthase in *Lolium* sp. (Duhoux and Délye, 2013).

Among the factors that have direct interference in a cropping process, the presence of weeds stands out due to the competition for resources. The competition may be either intra or interspecific, when one or more resources required to the development and growth are limited to meet the needs of all individuals present in the environment (Radosevich et al., 2007).

In the present study, we assessed the stability of candidate reference genes in order to identify the most appropriate genes for the normalization of the transcription in rice and red rice competing under different nitrogen levels and demonstrate the effectiveness of the reference gene selected by the expression of the cytosolic ascorbate peroxidase (*OsAPX2*) which is a key enzyme in the antioxidant metabolism.

## MATERIAL AND METHODS

### Plant material and experimental conditions

We used the rice cultivar IRGA 424 and a red rice biotype, varying the ratio of plants per pot (with no (100:0) and with (50:50) competition). The soil was added with different nitrogen dosages (0, 120, and 240 kg N ha<sup>-1</sup>). Sixty days after germination, we collected the shoots of plants, stored at -80 °C temperature up to the total RNA extraction and molecular analysis.

### Total RNA extraction and cDNA synthesis

The total RNA was extracted from the leaves of rice and red rice using the reagent PureLink™ (Plant RNA Reagent – Invitrogen™), as recommended by the manufacturer. The cDNAs were obtained by using the commercial kit SuperScript First-Strand System for real-time RT-qPCR (Invitrogen™), following the manufacturer's recommendations. The quantity and quality of RNA were evaluated by agarose gel electrophoresis 1% (p/v). The quantity and purity of RNA were determined by using a spectrophotometer NanoDrop™ 2000 (Thermo Scientific) with 260/280 nm ratio in the 1.9 to 2.2 interval and 260/230 nm around 2.0 considered as acceptable for use in real-time RT-qPCR.

### Determination of reference and target genes and Real-Time PCR conditions

For reference genes, we selected 11 primer pairs, mentioned in literature in studies on rice and used as internal control in real-time RT-qPCR analyses and that supposedly showed no significant variation among the treatments analyzed (Table 1). In order to validate the reference gene, we used the ascorbate peroxidase gene (*OsAPX2- EC1.11.1.11*), Forward (5'AGAGTCAGTACGATCAAGAC3') and Reverse (5'TCTTGACAGCAAATAGCTTG3') (Zhang and Hu, 2009).

For the amplification reaction, we used a total volume of 12 µL, containing 6.25 µL LightCycler® 480 SYBR Green I Master (Roche Applied Science), 0.5 µM primer (10 mM), 1 µL cDNA (0.2 µg) and water in an amount to complete the final volume. The amplification conditions were as follows: one cycle of 95 °C (5 min), followed by 45 cycles of denaturation at 95 °C (20 s), 60 °C (15 s), and 72 °C (20 s), interrupted by the dissociation curve with denaturation at 95 °C (5 s), cooling at 70 °C (1 min) and gradually heating at 0.11 °C steps up to 95 °C and cooling at 40 °C (30 s), using the system LightCycler 480 (Roche Applied Science). All reactions were carried out in triplicate for each cDNA sample. The amplicon purity was determined when a single melting peak was reached.

### Effectiveness and analysis of stability expression of reference genes

The PCR effectiveness was obtained from four serial dilutions of cDNA (1:1, 1:5, 1:25, and 1:125) to generate the standard slope for each pair of primer tested. The value of E was estimated by the equation  $E = 10^{(-1/\text{slope})}$  (Rasmussen, 2001), and values of effectiveness between 1.8 and 2.2 were considered as acceptable for reference genes.

To classify and determine the performance of each reference gene were used the average Ct values for each test sample obtained by each reaction cycle in real-time RT-qPCR. Data were subjected to analysis of variance, using the Statistical Analysis System - Winstat - Version 2.0

**Table 1** - Relation of reference primers used for real-time RT-qPCR in rice and red rice in response to the stress caused by competition and nitrogen levels

Gene (reference)	F (5'-3')	R (5'-3')	Efficiency rice	Efficiency red rice
<i>ACT11</i> (Zhang and Hu, 2009)	CAGCCACACTGTCCCCATCTA	AGCAAGGTCGAGACGAAGGA	2.44	2.36
<i>β-Tubulina</i> (Zhang and Hu, 2009)	GCTGACCACACCTAGCTTTGG	AGGGAACCTTAGGCAGCATGT	2.87	2.83
<i>Eef-1a</i> (Zhang and Hu, 2007)	TTTCACTCTTGGTGTGAAGCAGAT	GACTTCCTTACAGATTTCATCGTAA	1.84	2.15
<i>UBC-E2</i> (Jain et al., 2006)	CCGTTTGTAGAGCCATAATTGCA	AGGTTGCCTGAGTCACAGTTAAGTG	2.14	2.05
<i>eIF-4-a</i> (Jain et al., 2006)	TTGTGCTGGATGAAGCTGATG	GGAAGGAGCTGGAAGATATCATAGA	2.13	2.13
<i>UBQ10</i> (Jain et al., 2006)	TGGTCAGTAATCAGCCAGTTTGG	GCACCACAAATACTTGACGAACAG	1.83	1.82
<i>UBQ5</i> (Jain et al., 2006)	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT	2.19	2.20
<i>GAPDH</i> (Jain et al., 2006)	AAGCCAGCATCTATGATCAGATT	CGTAACCCAGAATACCCTTGAGTTT	2.06	2.10
<i>TIP41 - Like</i> (Caldana et al., 2007)	GTTTGGATGAACCCGCAA	GGCAACAAGTCAATCCGATC	1.50	2.30
<i>Cyclophilin</i> (Caldana et al., 2007)	CCACCATCACAGATCGGATCTT	CGTAACCCAGAATACCCTTGAGTTT	2.17	2.03
<i>18S rRNA</i> (Jain et al., 2006)	CTACGTCCCTGCCCTTTGTACA	GCAACAAGGTCAATCCGATC	2.20	2.14

(Machado and Conceição, 2003). Were considered stable those genes with lower standard deviation and coefficient of variation. At the same time, we observed changes in the expression levels both in the crop and in the weed, using the web-based tool RefFinder ([www.leonxie.com/referencegene.php](http://www.leonxie.com/referencegene.php)) which integrates all four software algorithms, GeNorm, NormFinder, BestKeeper, and comparative delta-Ct method. The detailed calculations for each one of the methods are described in Chen et al. (2011). The mean Ct value of each sample for each primer was used as input data on the website, and the Ct value belonging to the crop and the weed were analyzed altogether.

### Validation of reference genes

In order to ensure the reliability of the potential reference gene, the expression profile of *OsAPX2* was measured and normalized with the most stable reference gene as determined by RedFinder and the analysis of variance. The amplification conditions by real-time RT-qPCR were the same as abovementioned. The relative expression of data were calculated using the formula  $QR=2^{-\Delta CT}$ , modified from that proposed by Pfaffl et al. (2002), which was subjected to the analysis of variance ( $p \leq 0.05$ ) to test possible variations for the factors nitrogen, competition, and plant (rice and red rice), in isolation, as well as the interaction between both factors according to Tukey's test ( $p \leq 0.05$ ).

## RESULTS AND DISCUSSION

### Effectiveness and stability of reference genes

The amplification effectiveness of reference genes was individually calculated from the logarithm (Log) of cDNA dilutions for the crop and the weed (Table 1). The most appropriate dilution for the sample amplification was 1:25 subsequently used to validate the target gene. The efficiency ranged from 1.5 to 2.87 for rice and from 1.82 to 3.45 to red rice. For both competitors, the endogenous genes 18S ribosomal RNA (*18S*), cyclophilin, eukaryote elongation factor 1- $\alpha$  (*Eef-1a*), eukaryote initiation factor 4- $\alpha$  (*eLF-4a*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ubiquitin-enzyme conjugate E2 (*UBC-E2*), ubiquitin 5 (*UBQ5*), and ubiquitin 10 (*UBQ10*) had their effectiveness as expected (between 1.8 and 2.2), and they were used to test the stability and, afterwards, the most stable one was used along with the target gene. On the other hand, the actin (*ACT*), beta tubulin ( $\beta$ -*Tubulin*), and aquaporin (*TIP41*) genes had effectiveness other than expected.

For the study of the stability, we analyzed the coefficient of variation (CV%), standard deviation (SD), and means (X) of reference genes of rice and red rice which had effectiveness between 1.80 and 2.20. For the crop, it was noticed that *UBC-E2*, *UBQ10*, and *eLF-4a* had the lowest CV (3.39, 4.14, and 3.45, respectively) and SD (0.94, 1.03, and 1.08, respectively) values, whereas for the weed the genes *UBC-E2*, *UBQ5*, and *eLF-4a* had lower CV (1.34, 1.57 and 2.18, respectively) and SD (0.38, 0.42 and 0.60, respectively) values, indicating higher stability expression (Table 2). In order to evaluate the stability expression of reference genes, in addition to the analysis of variance,

**Table 2** - General mean (M), Variation coefficient (VC%), and Standard deviation (SD) of eight endogenous genes in rice and red rice

		<i>18S</i>	<i>Cyclophilin</i>	<i>Eef-1a</i>	<i>Elf-4a</i>	<i>GAPDH</i>	<i>UBC-E2</i>	<i>UBQ5</i>	<i>UBQ10</i>
X	Rice	18.08	29.92	32.01	31.30	27.41	28.79	27.00	32.92
	Red rice	18.61	30.00	29.94	30.33	26.24	28.63	26.90	24.52
VC%	Rice	10.40	4.49	11.69	3.45	8.99	3.29	5.44	4.14
	Red rice	9.41	2.63	2.51	2.18	2.96	1.34	1.57	3.11
SD	Rice	1.88	1.34	3.74	1.08	2.46	0.94	1.35	1.03
	Red rice	1.75	0.78	0.75	0.60	0.77	0.38	0.42	0.76

we have also calculated and compared the mean stability expression (M) using the software geNorm, NormFinder, Bestkeeper, and comparative delta-Ct method through the web-based tool which provides stability rankings. The lower the geometric mean, the higher the stability expression of the reference gene, and M values exceeding the cut-off value of 1.5 are not considered to be stable among treatments (Chen et al., 2011).

The determination of the normalizing gene appropriate for the study of gene expression is the first step that allows the analysis of the relative expression pattern of interest and target genes in a given experiment, providing more reliability to the results obtained. The software GeNorm is based on the principle that the ratio between the expressions of two reference genes is supposed to be frequent across different experimental conditions and/or organs/tissue. The value of M is determined as the average variation of the gene compared to the other ones tested. Based on M values calculated for the eight candidate normalization genes for rice competing with red rice, it was observed that *Cyclophilin* (M=1.27) and *UBC-E2* (M=1.27) were the most stable genes and *18S* (M=1.67) and *GAPDH* (M=1.85) as the most variable ones (Figure 1A). For red rice, the genes *eLF-4a* (M=0.70) and *UBC-E2* (M=0.70) were the most stable whereas *UBQ10* (M=1.01) and *18S* (M=1.37) were the least stable ones (Figure 2A).

According to the algorithm of NormFinder software which analyzes both intra and intergroup variations, the candidate genes in rice presenting the lowest M value are *cyclophilin* (0.70) and *UBC-E2* (M=0.81). The highest M values were for *18S* (M=1.85) followed by *GAPDH* (M=2.11) (Figure 1B). The same was observed in red rice and the genes *UBC-E2* (M=0.41) and *cyclophilin* (M=0.49) were the most stable ones, whereas *UBQ10* (M=1.23) and *18S* (M=2.34), the least stable ones (Figure 2B).

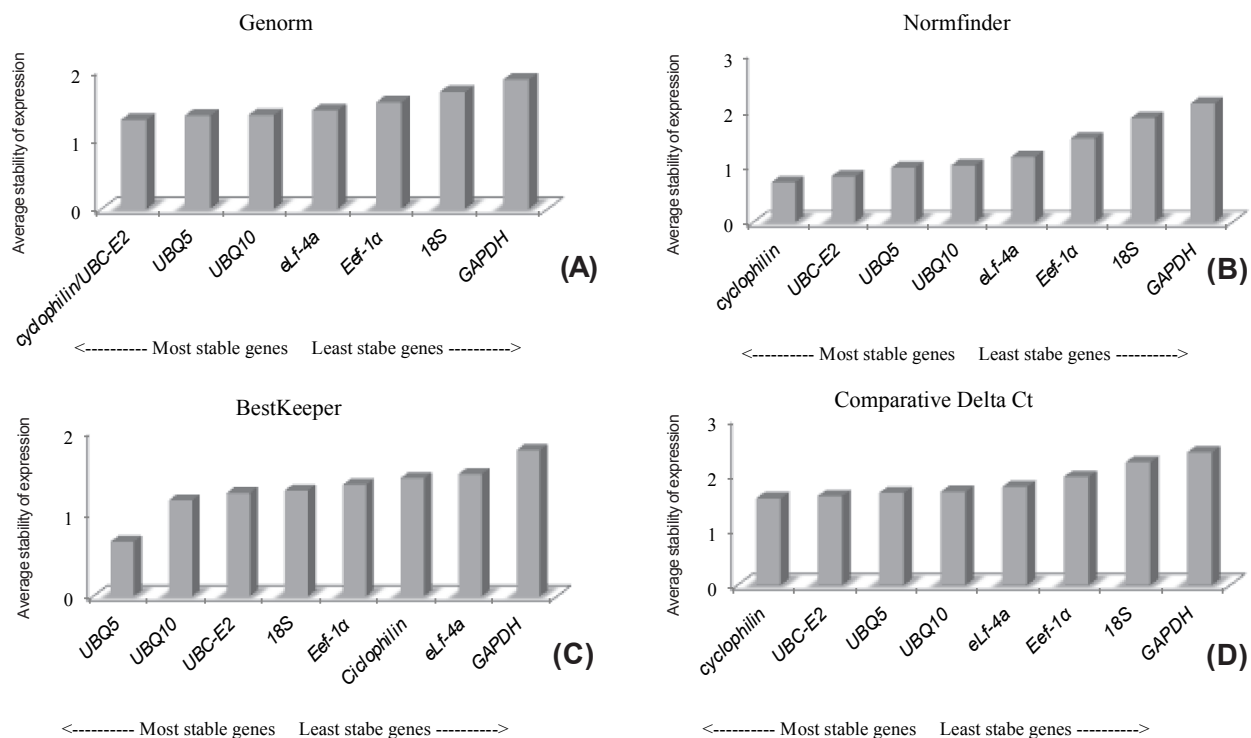
The BestKeeper software algorithm calculates the standard deviation (SD) and establishes the value 1 as the cut-off (SD = 1), being considered as stable genes those with SD value lower than 1 (SD<1) and unstable those with values higher than 1 (SD>1). The analysis indicated only *UBQ5* as the most stable reference gene in rice presenting SD = 0.66, whereas the other genes were considered unstable (Figure 1C). For red rice, the most stable genes were *UBQ5* (M=0.65), *UBC-E2* (M=0.77), *cyclophilin* (M=0.83), *eLF-4a*, and *GAPDH* (M=0.95), and the others did not present stability (Figure 2C).

According to the comparative delta Ct method, in rice, the genes *cyclophilin* (M=1.56) and *UBC-E2* (M=1.60) were the most stable and presented a stability expression order close to the GeNorm algorithm for the three first candidate normalization genes, even though it has shown different M values for each gene (Figure 1D). In red rice, the same genes, *cyclophilin* (M=1.09) and *UBC-E2* (M=1.15), were the most stable ones, whereas *UBQ10* and *18S* (M=1.54 and 2.45, respectively) were the least stable ones (Figure 2D).

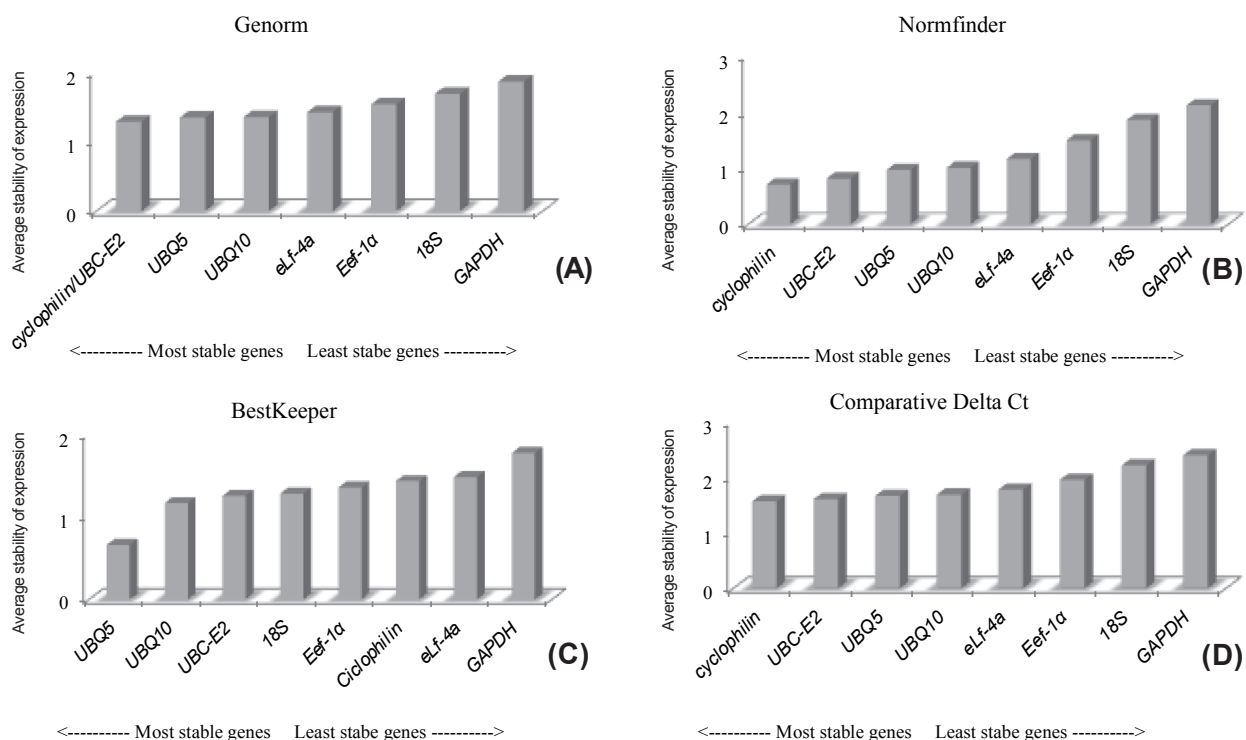
According to the general acceptance criteria, the ideal reference gene is stably expressed (or, at least, only slightly varying in the expression) among the sets of samples investigated and has an expression level compared to the target gene (Andersen et al., 2004). Appropriate reference genes have already been identified for many crops especially for model plants (Czechowski et al., 2005), however, a few reference genes have been validated for weeds because most of them are species with no genomic information available. Also, the extrapolation from other species even in taxa closely related is not indicated since the expression of putative reference genes varies between different sets of organs and different experimental conditions (Hruz et al., 2011), i.e., there is no universal reference gene.

Based on the results of effectiveness, analysis of variance and mean stability according to the combination of the algorithms GeNorm, NormFinder, Bestkeeper, and Comparative Ct method, the normalizing gene *UBC-E2* was selected for rice and red rice. One of the three main components of the ubiquitination system is the ubiquitin-conjugating enzyme (*UBC-E2*) which bounds the ubiquitin (Ub) to the substrate (Unver et al., 2012). The ubiquitination is involved in many important processes such as plant growth and development, hormonal regulation, flowering, and responses to biotic and abiotic stresses (Dreher and Callis, 2007).

In the analysis of ten normalizing genes as internal control for gene expression studies on 25 rice samples, it was found that *UBQ5* and *Eef-1a* were the most stably expressed, whereas



**Figure 1** - Average stability expression (M) according to the algorithm GeNorm (A), NormFinder (B), BestKeeper (C), and comparative delta-Ct method (D) of eight candidate normalization genes in rice competing with red rice in different ratios and nitrogen levels.



**Figure 2** - Average stability expression. (M) according to the algorithm GeNorm (A), NormFinder (B), BestKeeper (C), and comparative delta-Ct method of eight candidate normalization genes in red rice competing with rice in different ratios and nitrogen levels.

*UBQ10* exhibited the least stable expression in different tissues or cell types at different developmental stages (Jain et al., 2006). This result differs from that observed in this study, since the gene *UBC-E2* was the most stable one, presenting low CV, SD, and M values, indicating higher stability expression both for rice and red rice. Nevertheless, Li et al. (2012) by submitting rubber tree (*Hevea brasiliensis*) to different conditions found the gene *UBC* as the most stable of several tested. The reference genes are regulated differently in different plant species and may exhibit distinct expression patterns. Therefore, a reference gene presenting stable expression in an organism may or may not be appropriate for the normalization of gene expression in another organism in a set of conditions and needs to be validated before its use (Jain et al., 2006). Several studies have shown that the expression of a same reference gene may vary in certain situations (Thellin et al., 1999). This may be partly explained by the fact that reference genes are not only implicated in the metabolism of basal cells, but also taking part in other cell functions (Singh and Green, 1993; Ishitani et al., 1996).

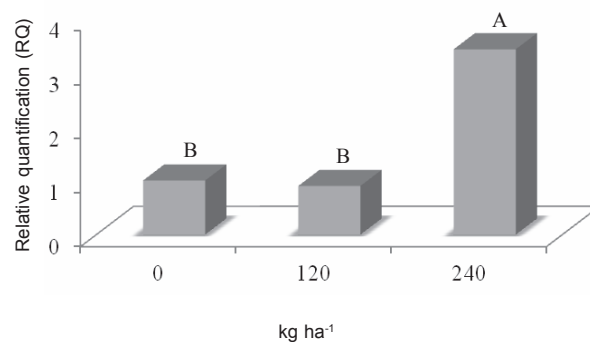
### Validation of the reference gene – Expression of *OsAPX2*

For the gene *OsAPX2* in rice competing with red rice, there was no interaction between the factors tested, only an effect of nitrogen dose with an increased expression in the dosage 240 kg ha<sup>-1</sup> nitrogen (QR=3.43), differing from 0 kg ha<sup>-1</sup> (QR=1.00) and 120 kg ha<sup>-1</sup> (QR=0.90) (Figure 3).

In red rice, there was an interaction between the factors tested for the expression of the gene *OsAPX2* showing higher expression of the gene in the dosage 240 kg ha<sup>-1</sup> and 50% ratio in comparison to the monoculture (QR=3.34), and no differences between the ratios in the dosages 0 kg ha<sup>-1</sup> and 240 kg ha<sup>-1</sup> (Figure 4).

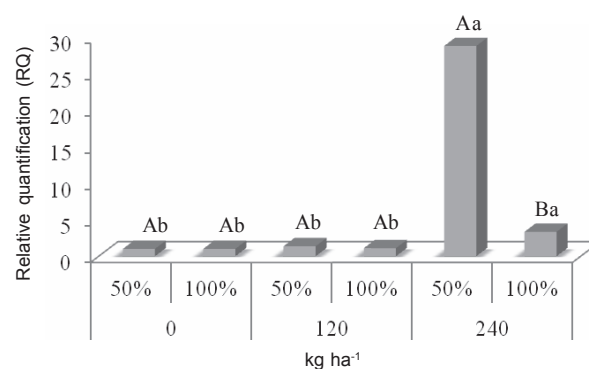
The APXs are the main peroxidases removing H<sub>2</sub>O<sub>2</sub> inside cells and act along with other enzymes which play a role in the ascorbate-glutathione cycle (Foyer and Noctor, 2000). The APX may have an increased activity in response to environmental stresses such as salinity, low temperatures, metal poisoning, drought, high temperatures, ozone, high luminous intensity, pathogens, among others, as reported for different plant species (Yoshimura et al., 2000; Mittova et al., 2004; Sharma and Dubey, 2004). When rice and red rice were in an interspecific competition, subjected to the dosage of 240 kg ha<sup>-1</sup> nitrogen, the expression of the gene *OsAPX2* increased, indicating a possible greater oxidative stress. According to Kandlbinder et al. (2003), the excess of nitrogen could promote an increase of APX in rice.

Based on results of effectiveness, analysis of variance and Reference Finder, the normalizing gene *UBC-E2* was selected for rice and red rice in competition. The expression of the gene *OsAPX2* has proven to be effective in validating the candidate reference gene. The study is of great importance in other gene expression analyses aiming at understanding the mechanisms of competitive stress tolerance in rice and red rice plants.



Upper-case letters are comparisons of nitrogen doses.

**Figure 3** - Relative quantification of *OsAPX2* gene expression in rice competing with red rice under different nitrogen doses.



Upper-case letters are comparisons of plant ratios within nitrogen doses and lower-case letters are comparisons of nitrogen doses within plant ratios.

**Figure 4** - Relative quantification of *OsAPX2* gene expression in red rice competing with rice in different ratios and nitrogen doses.

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