Australian Journal of Crop Science AJCS

AJCS 10(5):749-757 (2016) ISSN:1835-2707
DOI:10.21475/ajcs.2016.10.05.p7441

Expression of genes in cultivated rice and weedy rice in competition

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Abstract

The competition for resources such as light and nitrogen between red rice and cultivated rice can trigger responses in plants that interfere with growth and productivity as well as the expression of genes related to competition-induced stress. Due to its sensitivity, accuracy, and specificity, real-time reverse transcription PCR (RT-qPCR) is an important technique for analyzing differences in gene expression. In this study, we quantified the relative expression levels of genes involved in nitrogen assimilation (*OsAMTs*, *OsGS2* and *OsNADH-GOGAT2*) and light capture (*OsPIL1*, *OsCRY2* and *OsCAB1*) in cultivated and red rice in competition under different concentrations of nitrogen (0, 120 and 240 kg ha^{-1} of nitrogen). Interspecific competition in rice increased the expression of certain genes responsible for assimilating nitrogen (*OsNADH-GOGAT2* and *OsAMT3;1*), and intraspecific competition in red rice also increased the expression of *OsGS2*. With the interspecific competition, both rice and red rice exhibited increased expression of genes responsible for capturing light, such as *OsCRY2* and *OsCAB1*. With intraspecific competition, red rice showed increased *OsPIL1* expression. Additionally, higher doses of nitrogen increased the expression of genes responsible for assimilating nitrogen and capturing light in both cultivated and wild rice species.

Keywords: interference, real-time reverse transcription PCR, *Oryza sativa*.
Abbreviations: AMT_ammonium transporter; GS2_glutathione synthetase isoform; GOGAT2_glutamine 2-oxoglutarate aminotransferase; CRY2_cryptochrome 2; CAB1_ chlorophyll a/b; PIL1_factor-binding protein that interacts with phytochrome; DAE_days after emergence; RQ_Relative quantification.

Introduction

Rice crops have great social, economic and cultural value because rice is the staple food for over half the world's population. Among rice-producing countries, Brazil ranks ninth and produces 13 million tons (FAO, 2014).

Although Brazil occupies a prominent position in global rice production, it is not sufficiently productive to be considered ideal for this crop. This concern partially stems from unsatisfactory weed control, particularly for red rice, which is the main weed species, and inadequate management of fertilizers, especially nitrogen. Analyzing these two critical factors together, rice competes for nitrogen with red rice, which reduces nutrient availability to the crop and generates stressful conditions. One study demonstrated that rice plants in competition with red rice biotypes show a reduced leaf area and less dry matter in the shoots with low soil nitrogen levels (Chauhan and Johnson, 2011). However, despite the potential for verifying these responses based on morphophysiological variables, the effects of competition on plant gene expression have not yet been elucidated.

Nitrogen uptake and assimilation pathways in rice are well documented (Suenaga et al. 2003; Tabuchi et al. 2007; Yuan et al. 2007). Primary assimilation of $NH4^+$ occurs in both shoots and roots, and $NH4^+$ is then incorporated into organic molecules by glutamine synthetase (GS) and glutamate synthase (NADH-GOGAT). In *Arabidopsis thaliana* (a model dicot), the nitrogen transporter gene *AtAMt1.1* plays important roles in nitrogen uptake and root growth and development under nitrogen starvation conditions (Tony et al. 2006; Engineer and Kranz 2007). In rice, expression of *OsAMT1;1* is clearly upregulated in response to nitrogen starvation (Li and Shi 2006; Loqué et al. 2006; Mayer and Ludewig 2006); in other plants (e.g., maize and alfalfa), GS and NADH-GOGAT have been shown to play important roles in nitrogen assimilation (Chichkova et al. 2001; Hirel et al. 2001).

In addition to competing for nitrogen, plants also compete for light because in nature, leaves often have to cope with reduced light intensities and altered light qualities resulting from shading by the canopy or by neighboring plants (Terashima et al. 2005). Indeed, light is one of the most important environmental factors controlling various stages of plant growth and development, from seed germination to flowering. Plants perceive light signals via several types of photoreceptors, including phytochromes and cryptochromes, allowing them to detect changes in the environment. Phytochromes predominantly absorb red (600–700 nm) and far-red light (700–750 nm), whereas cryptochromes respond to blue and UV-A light (320–500 nm) (Prasada et al., 2012).
In rice, cryptochromes have multiple functions, and their multiple intracellular localization patterns correspond to these functions. Cryptochromes cause various photomorphogenic changes in response to blue-light signals, such as cotyledon

expansion and inhibition of hypocotyl elongation, as well as anthocyanin accumulation and chalcone synthetase gene expression (Cashmore et al., 1999; Lin, 2002; Lin and Shalitin, 2003). A study using transgenic rice plants overexpressing one of the three isoforms suggested that all of the cryptochromes in rice plants mediate B light perception to suppress elongation of leaf sheaths and blades (Hirose et al. 2006).

Phytochrome-interacting factor-like protein, *OsPIL1*, acts as a key regulator of reduced internode elongation in rice under drought conditions. In rice seedlings grown under nonstressed conditions with normal light/dark cycles, the level of *OsPIL1* mRNA oscillates in a circadian manner with peaks in the middle of the light period; however, *OsPIL1* expression is inhibited during the light period under drought stress (Todaka et al., 2012). The expression of many light-inducible genes is regulated at the transcriptional level. The nuclear-encoded photosynthesis-related gene for chlorophyll a/b binding protein (CAB) is expressed at high levels upon exposure to light (Gilmartin et al., 1990, Manzara et al., 1991; Terzaghi and Cashmore, 1995). The period of CAB expression in Arabidopsis, for example, is approximately 24.5 h in constant light but extends to 30 h or more in constant darkness (Millar et al., 1995.)

Due to it sensitivity and reproducibility in analyzing transcript levels, RT-qPCR (real-time quantitative reverse transcription polymerase chain reaction) has been widely used to validate the gene expression (Gachon et al., 2004). To our knowledge, this is the first comprehensive analysis of gene expression related to nitrogen assimilation and light gathering in rice and red rice competing under different levels of nitrogen. Knowledge of the gene expression levels of plants in competition for nitrogen may aid in developing management strategies that increase nutrient utilization by cultivated rice, which may lead to reduced fertilizer use. In addition, such studies provide a basis for the breeding of plants with greater competitive ability, which may help to improve productivity.

Thus, the aim of this study was to evaluate the expression levels of genes involved in plant responses in intraspecific and interspecific competition as well as at different levels of nitrogen.

Results and Discussion

Expression of genes responsible for nitrogen assimilation

The RT-qPCR amplification specificity was confirmed by the presence of a single peak in the dissociation curve (melting) after 45 cycles.

In evaluating the target gene *OsAMT1;1* in rice and red rice, we did not detect a statistically significant interaction among or main effect from the factors nitrogen and plant proportions. As the *OsAMT1;1* gene is expressed in roots and shoots, especially in older leaves (Su-Mei et al., 2012), it was impossible to verify the difference in expression between the nitrogen and competition samples because we used young leaves in these analyses.In rice, the *OsAMT3;1* gene did not exhibit a statistically significant interaction or main effect for the factors nitrogen and plant proportions. Red rice exhibited an interaction between the factors tested for expression of the gene *OsAMT3;1*, and at the nitrogen dose 120 kg ha^{-1}, the gene was upregulated by 50% (RQ = 6.39) compared with a monoculture (RQ = 0.70); however, we did not observe differences in expression for nitrogen doses of 0 kg ha^{-1} and 240 kg ha^{-1} (Fig. 1). The AMT proteins in plants are located in the plasma membrane, which suggests that they play a role in ammonium uptake in plant cells (Shi et al., 2010). When we supplied nitrogen to plants at the appropriate dose (120 kg ha^{-1}), expression of AMT mRNA increased in red rice plants. However, in comparison with the rice culture at 240 kg ha^{-1}, expression was lower in both the monoculture and competitive condition, likely due to insufficient levels of nitrogen to both species because, other than nitrogen, the plants received plenty of nutrients.

Unlike *OsAMT1;1*, the *OsAMT3;1* gene is expressed in the shoot, especially in young leaves (Su-Mei et al, 2012.). The same authors found that the rice genes *OsAMT1;1* and *OsAMT3;1* exhibited increased expression in the absence of nitrogen for 48 hours but that the expression of *OsAMT1;1* decreased when $NO3^-$ was replenished.

In competition with red rice, the rice *OsGS2* gene did not show an interaction between the examined factors, with an effect on only nitrogen rate, and the expression increased at 240 kg ha^{-1} of nitrogen (RQ = 1.79 vs. 0 kg ha^{-1} (RQ = 1.00) and 120 kg ha^{-1} (RQ = 0.99)) (Fig. 2A). For red rice, the factors showed an interaction; the highest expression of *OsGS2* was observed under intraspecific competition at 240 kg ha^{-1}; the proportions did not differ at other doses (Fig. 2B). This increased expression at a higher dose may be associated with high levels of available nutrients in the environment because GS2 is the main glutathione synthetase isoform found in the leaves, and its overexpression is associated with maintaining photosynthetic capacity, photorespiration in plants and nitrogen assimilation (Shi et al., 2010). Furthermore, we did not observe differences upon comparing the doses with equal proportions of competing plants; however, the red rice monoculture showed its highest level of expression at the highest dose of nitrogen examined.

The *OsNADH-GOGAT2* gene in cultivated rice showed a 50% effect (RQ = 1.08) of intraspecies competition and a 100% effect (RQ = 0.53) of interspecies competition, regardless of the nitrogen dose used (Fig. 3). This increased expression during interspecies competition is likely due to an attempt to capture more nitrogen than the surrounding plants (weeds) because this nutrient is essential for maintaining the physiological functions of the plant. The wild red rice did not show a statistically significant change in gene expression under either treatment, and the expression of *OsNADH-GOGAT2* was independent of the nitrogen dose as well as of intraspecific and interspecific competition. *OsNADH-GOGAT2* is a key enzyme for primary nitrogen assimilation and re-assimilation in plants and is expressed in only the leaf and leaf sheath (Tabuchi et al., 2007). Deleting this gene reduced the nitrogen assimilation rate in rice and produced nitrogen-deficient phenotypes, such as fewer roots and shoots, even under hydroponic conditions with sufficient nitrogen (Lu et al., 2011).

Expression of genes involved in light perception

We evaluated the *OsPIL1* gene in cultivated rice and did not observe a statistically significant main effect or interaction with the nitrogen dose for the plant factors and ratios. However, although the red rice *OsPIL1* gene showed similar responses to nitrogen doses, under monoculture conditions, this gene exhibited higher expression (RQ = 1.59) than in cultivated rice (RQ = 0.94), indicating that intraspecific competition can stress red rice plants and reduce their height (Fig. 4).

Light is a critical resource for rice competition with red rice (Agostinetto et al., 2001); light is perceived by specific

Table 1. Sequences of target genes used for RT-qPCR in cultivated rice and red rice in response to nitrogen and stress caused by competition.

Primer	F (5'-3')	R (5'-3')	Efficiency rice	Efficiency red rice
OsAMT1;1	TCTCTTCTACGGGCTCAAGAAGC	CAAATTTATGACGTGACGATCGAGA	2.00	1.90
OsAMT3;1	CTCCCGCAGACGACGCAGTT	GCCGACGGTGTAGGAGAAGGTG	2.10	1.88
OsGS2	AGAACTTGGACGATGAATCGG	CATTTTATTTCGAGGGAAGG	2.00	2.07
OsCRY2	GATGCAACAAATCAAGTGGG	CGCTCCTCATCTACTTTCCA	2.10	1.89
OsCAB1	TATGGACCTGACCGTCCCAA	ATCAACTCCAGCTCCCTGTTC	2.10	2.16
OsNADH-GOGAT2	CCTGTCGAAGGATGATGAAGGTGAAACC	TGCATGGCCCTACTATCTTCGCATCA	2.08	2.10
OsPIL1	GCAAACAGTGCCACCACAGG	CTAAATTCCATCAGAGGTTGG	2.00	2.20

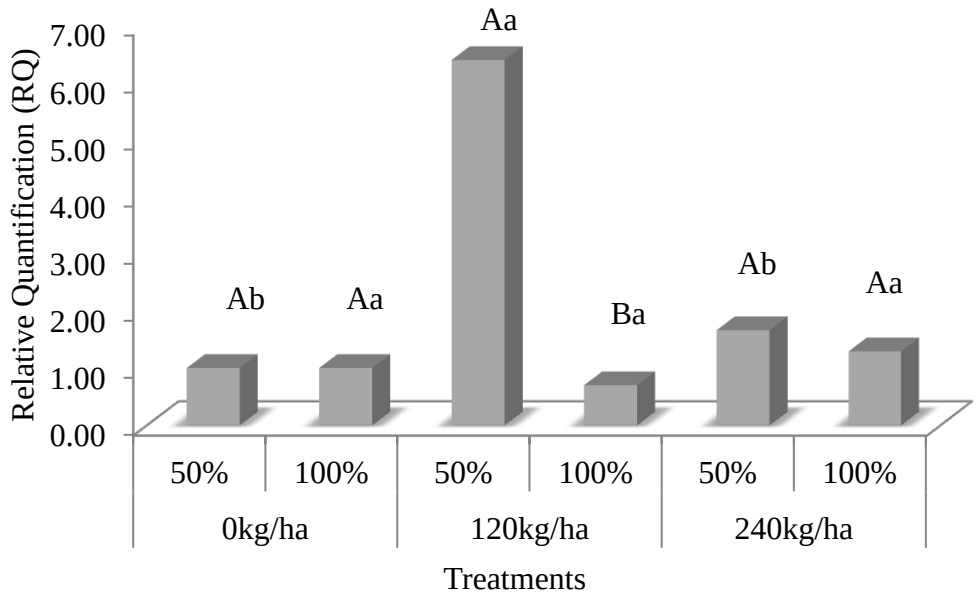


Fig 1. Relative quantification of the *OsAMT3;1* gene for red rice in competition with cultivated rice at different proportions and with different levels of nitrogen. The capital letters indicate a difference in the proportions of interspecies competition, and the lower case letters indicate a difference between the doses of nitrogen.

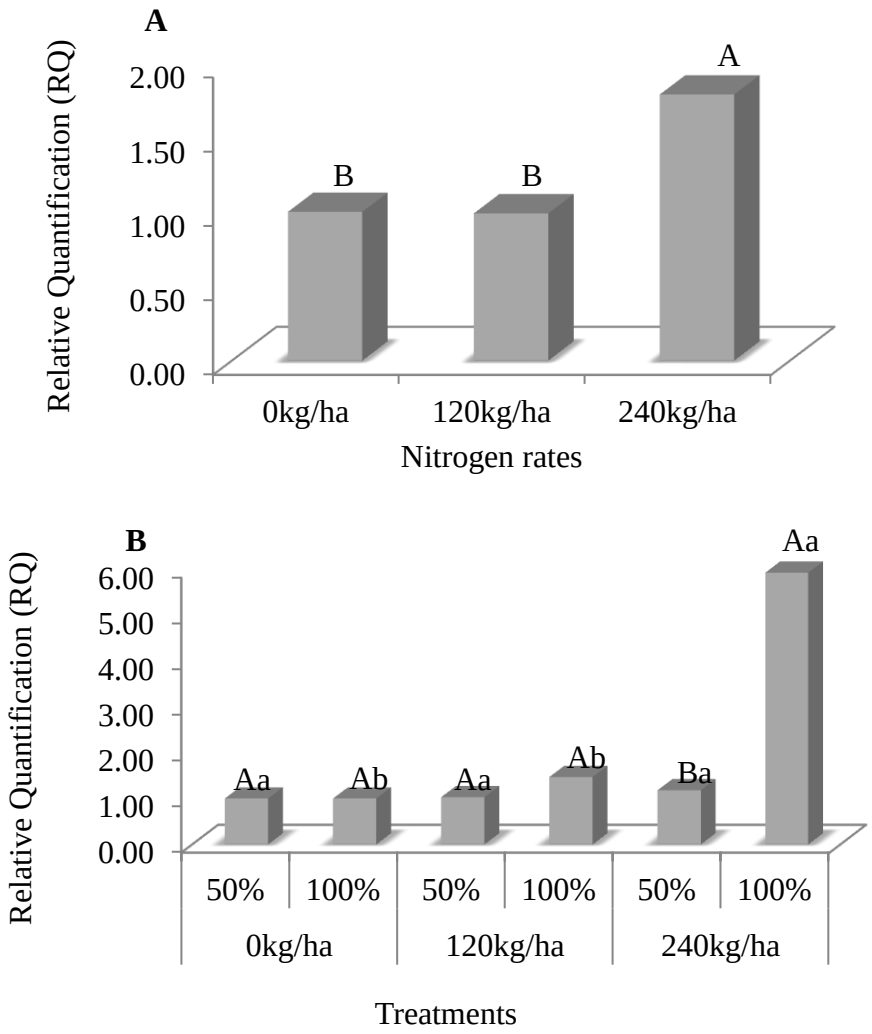


Fig 2. Relative quantification of the *OsGS2* gene for cultivated rice in competition with red rice under different levels of nitrogen (A) and for red rice in competition with cultivated rice at different proportions and under difference levels of nitrogen (B). The capital letters indicate differences in the proportions of interspecies competition, and the lowercase letters indicate differences between the doses of nitrogen.

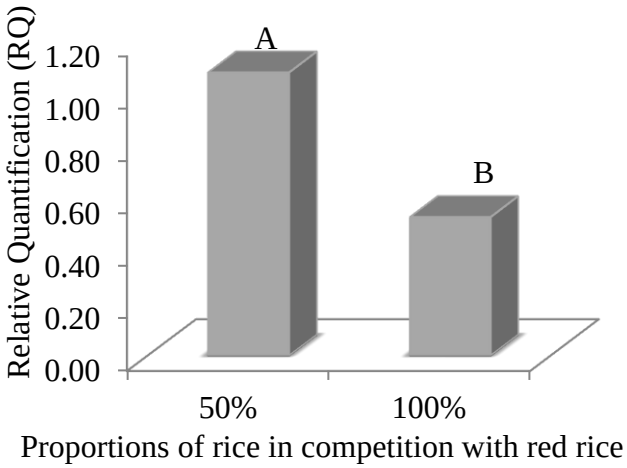


Fig 3. Relative quantification of the *OsNADH-GOGAT2* gene for different proportions of cultivated rice in competition with red rice. The capital letters indicate a difference between the plant proportions.

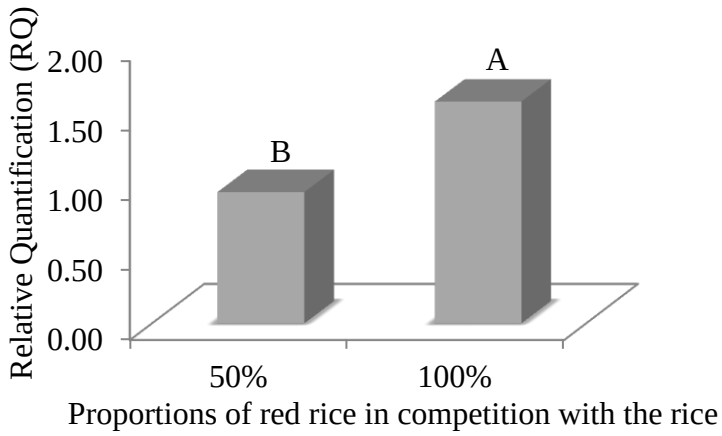
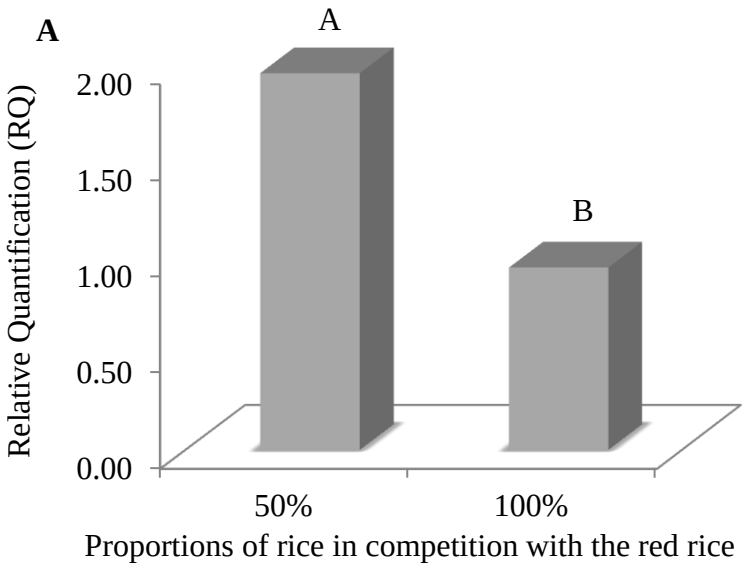


Fig 4. Relative quantification of the *OsPIL1* gene for different proportions of red rice in competition with cultivated rice. The capital letters indicate a difference between the plant proportions.



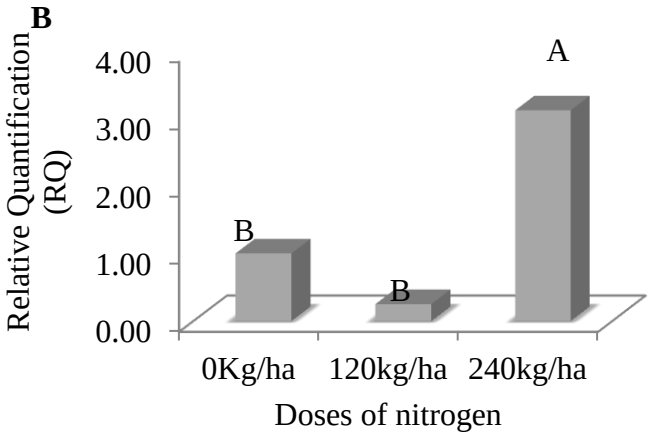


Fig 5. Relative quantification of the *OsCRY2* gene for cultivated rice in competition with red rice at different proportions (A) and different doses of nitrogen (B). The capital letters indicate differences between the proportions of plants or the doses of nitrogen.

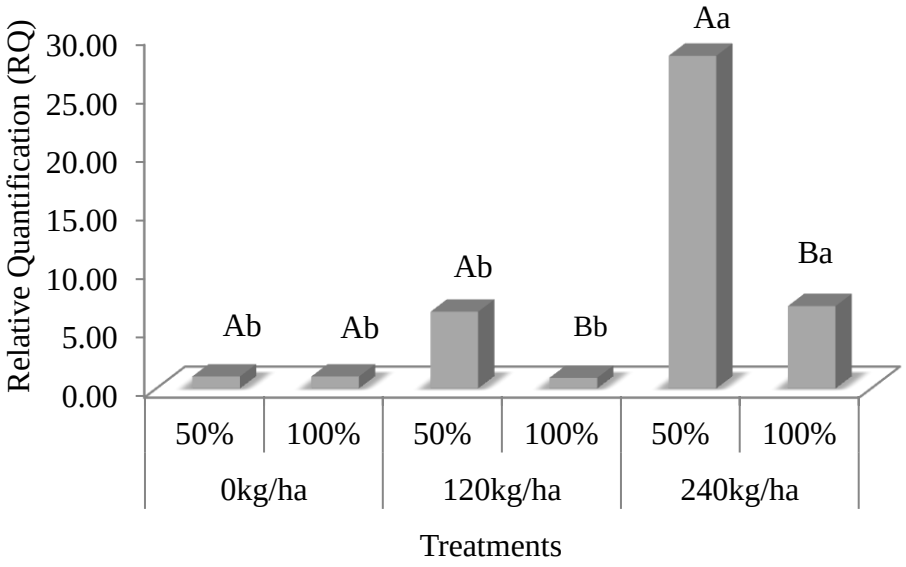


Fig 6. Relative quantification of the *OsCRY2* gene in red rice in competition with cultivated rice at different proportions and with different levels of nitrogen. The capital letters indicate differences between the proportions of competition, and the lower case letters indicate differences between the doses of nitrogen.

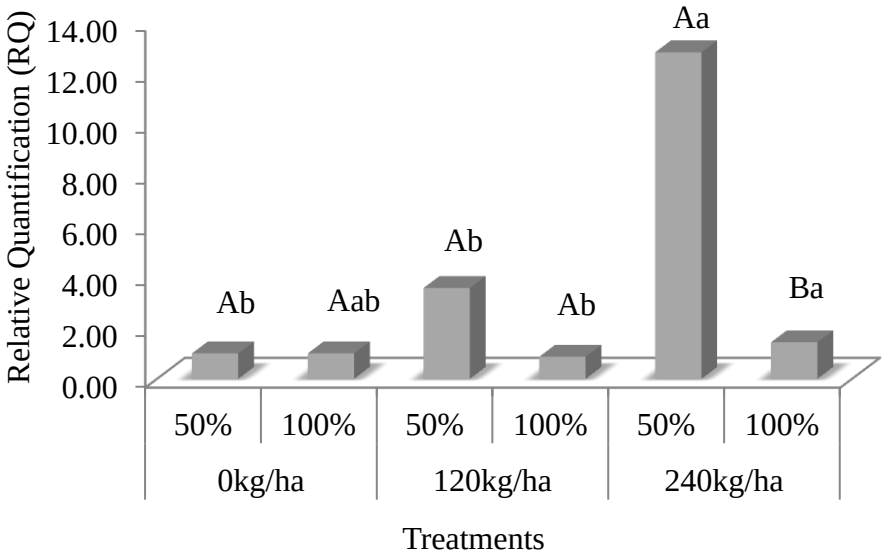


Fig 7. Relative quantification of the gene expression of *OsCAB1* for red rice in competition with cultivated rice at different proportions and with different levels of nitrogen. The capital letters indicate differences in the proportions of competition, and the lower case letters indicate differences between the doses of nitrogen.

photoreceptors, including phytochrome, cryptochromes, phototropins and UVB radiation receivers (Jiao et al., 2007), which induce photomorphogenic effects that alter the ability of plants to capture additional resources.

We measured expression of *OsCRY2* in cultivated rice and found no interaction among the proportions and nitrogen levels; however, each factor produced a significant difference. For the proportion experiments (Fig. 5A), cultivated rice in competition with the weed exhibited higher expression (RQ = 1.96) than under monoculture conditions (RQ = 0.95). However, among the doses of nitrogen (Fig. 5B), 240 kg ha^{-1} (RQ = 3.12) yielded the highest expression of *OsCRY2*. For red rice, the *OsCRY2* gene showed interactions among the factors, and this gene was

overexpressed under interspecific competition at 120 kg ha^{-1} and 240 kg ha^{-1}; however, without nitrogen fertilization, we did not observe a difference between the levels of competition (Fig. 6). Comparing the nitrogen doses, we observed the highest expression increase at 240 Kg ha^{-1},in interspecific competition. Cryptochromes recognize blue light signals and may produce various photomorphogenic responses, such as growth inhibition through cotyledon and hypocotyl elongation, as well as anthocyanin accumulation and chalcone synthase gene expression (Lin and Shalitin, 2003). For both the cultivated and wild rice species, gene expression of *OsCRY2* was greater under interspecies competition at higher nitrogen doses. Light is important for rice physiology and morphology because under 50% shading, the culture environment reduces the dry matter mass of the shoot by 57% (Chauhan, 2013), and this impairs productivity. These results are related to the negative effect of low light availability on photosystem II, the chlorophyll content and the enzyme RuBisCO, which reduces photosynthesis and induces ROS formation (Voleti and Singh, 1996).

The *OsCAB1* gene in cultivated rice did not show a statistically significant change in expression based on the dose of nitrogen supplementation, the degree of interspecies competition, or the interaction between those factors. However, red rice exhibited an interaction among the factors: at 50% and 240 kg ha^{-1}, the *OsCAB1* gene exhibited greater expression, but differences at other proportions were not observed (Fig. 7). The highest nitrogen dose tested increased gene expression independent of the proportion of plants. Interspecific competition and an increase in nitrogen increased *OsCAB1* expression (chlorophyll a/b) in wild rice. The shading culture may have generated interspecific competition, increasing gene expression; however, the monoculture did not generate competition for light.

One of the factors related to the photosynthetic efficiency of plants and, consequently, to growth and adaptability to various environments, is chlorophyll, which is present in all green plants. Chlorophyll is constantly synthesized and destroyed (photo-oxidation) in the presence of light, but its decomposition rate is highest under high light intensity; a balance is established at lower levels of light (Kramer and Kozlowski, 1979).

Intra- and interspecific competition

The rice cultivation results demonstrate that relative to those genes involved in nitrogen assimilation, the AMT genes did not differ between the degrees of competition and nitrogen levels examined, but the expression of *OsGS2* increased at 240 kg ha^{-1} independent of interspecific or intraspecific competition. Gene expression of *OsNADH-GOGAT2* increased under interspecific competition, independent of the nitrogen dose. For red rice, among the genes studied that are involved in nitrogen assimilation, *OsAMT3;1* exhibited differential expression under interspecific competition and at 120 Kg ha^{-1} of nitrogen, and *OsGS2* exhibited differential expression under intraspecific competition and at 240 ha^{-1}. These results demonstrate interspecific competition for nitrogen assimilation and show that *OsGS2* increased in both the cultivated and weed species, which may be due to the abundance of nitrogen available to both sets of plants.

Among the genes involved in light capture, for cultivated rice, only *OsCRY2* exhibited increased expression when the culture was placed under interspecific competition or with nitrogen fertilization at 240 kg ha^{-1}. In contrast, only *OsPIL1* showed increased expression in wild rice under intraspecific competition, regardless of the nitrogen dose; *OsCAB1* and *OsCRY2* showed increased expression under interspecific competition and at 120 or 240 kg ha^{-1} of nitrogen and 240 kg ha^{-1} of nitrogen, respectively.

Although it is important to understand the effects of limiting or excess resources on plants, previous research has clearly focused on evaluating only abiotic factors that act in isolation without considering the presence of competition, i.e., weeds (Gorantla et al, 2007), or has considered only the effect of competition to the exclusion of other environmental influences (Horvath et al., 2007). However, in the field, plants are typically exposed to multiple stresses simultaneously; thus, understanding plant behavior requires consideration of the potential interrelationships of these stresses. Identifying the gene expression responses in cultivated rice in competition with red rice associated with environmental factors provides an opportunity to more deeply investigate key issues in weed science.

Materials and Methods

Plant material and nitrogen application

The experiments were performed using a completely randomized design, which was arranged using a factorial where factor A was composed of different combinations of the rice cultivar IRGA 424 and a red rice biotype by varying the relative proportions of plants per pot (without (100:0) and (50:50) competition). Factor B consisted of nitrogen added to the soil (0, 120 and 240 kg ha^{-1} of nitrogen (N)). The plant population was 32 plants per pot, which is equivalent to 1.143 plants m^{-2} and was determined using a preliminary monoculture experiment.

The nitrogen was applied to dry soil at two stages, 15 days after emergence (DAE) and 53 DAE. One day after the first fertilizer application, the experimental units were flooded using levees. At 60 DAE, we collected the shoots and stored them at -80 °C until the total RNA extraction and molecular analysis.

RNA isolation and cDNA synthesis

Total RNA was extracted from the cultivated rice and red rice leaves using the PureLink™ reagent (Plant RNA Reagent - Invitrogen™) following the manufacturer's recommendations. The total RNA was digested with DNase I - Invitrogen™ to degrade the contaminant DNA. The RNA integrity was assessed using electrophoresis and 2% agarose gels. The quantity and purity of RNA were determined using a spectrophotometer NanoDrop™ 2000 (Thermo Scientific) with ratios for 260/280 nm ranging from 1.9 to 2.2 and for 260/230 nm at approximately 2.0, which is considered acceptable for qRT-PCR. The cDNA was generated using the commercial kit SuperScript First-Strand system for RT-PCR (Invitrogen™) in accordance with the manufacturer's recommendations.

Primer design and quantitative RT-qPCR analysis

The target gene sequences were selected from studies cited in the literature and from rice transcripts in the National Center for Biotechnology Information (NCBI). The genes selected were genes encoding ammonium transporters *(OsAMT1;1*, AF289477 and *OsAMT3;1*, AB083582); glutathione synthetase 2 (*OsGS2*, X14246); cryptochrome 2 (*OsCRY2*, AB103094), chlorophyll a/b (*OsCAB1*, D00642.1); glutamine 2-oxoglutarate aminotransferase (*OsNADH-GOGAT2*, AB274818) and phytochrome-interacting factor-binding

protein (*OsPIL1*, LOC_Os03g56950) (Table 1). The reference gene was an E2 ubiquitin-conjugating enzyme, (OsUBC-E2)-F (5'CCGTTTGTAGAGCCATAATTGCA3') and OsUBC-E2-R (5'AGGTTGCCTGAGTCACAGTTAA-GTG3') for rice and red rice, respectively; the primers for this gene were preliminarily selected from 11 pairs of primers, and it is reported in the literature as an internal control for rice research RT-qPCR analyses.

For the amplification reaction, we used a total volume of 12 µL containing 6.25 µL of LightCycler® 480 SYBR Green I Master (Roche Applied Science), 0.5 µM primer (10 mM), 1 µl cDNA (dilution 1:25, previously defined) and water to reach the final volume. The amplification conditions were in accordance with the manufacturer's instructions for the LightCycler 480 system (Roche Applied Science). The reactions were performed in triplicate for each cDNA sample. The amplicon was assumed to be pure when it produced a single melting peak.

Gene expression stability analysis

To verify the stability of the target genes, the changes in expression level were compared between the cultivated and wild rice species and among different levels of competition (50% and 100%) and nitrogen (no added nitrogen, 120 kg ha^{-1} and 240 kg ha^{-1}). The relative levels of expression (RQ) were quantified using the comparative cycle threshold (CT) method (Livack and Schmittgen 2001) and the equation $QR= 2^{-\Delta\Delta CT}$. The RQ were submitted to analysis of variance; the quantified average RQ for the nitrogen rates and competition factors were compared using Tukey's test with a significance level of $p \leq 0.05$.

Conclusions

RT-qPCR allowed us to analyze the expression of genes responsible for nitrogen uptake and light capture in cultivated rice and red rice plants in competition. For cultivated rice, interspecific competition increased the expression of genes responsible for nitrogen assimilation (*OsNADH-GOGAT2*) and light gathering (*OsCRY2*). However, the competitive interaction in red rice varied depending on the target gene and on the use of a higher nitrogen dose, which increased the expression of genes responsible for nitrogen assimilation and light capture in both cultivated and weed species of rice.

Acknowledgments

The authors are grateful for the financial support of DECIT/SCTIE-MS via CNPq and FAPERGS.

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