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Enzyme expression in *indica* and *japonica* rice cultivars under saline stress

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ABSTRACT. The southern State of Rio Grande do Sul (RS) is the main rice producer in Brazil with a 60% participation of the national production and 86% participation of the region. Rice culture irrigation system is done by flooding, which leads to soil salinization, a major environmental constraint to production since it alters the plants' metabolism exposed to this type of stress. The *indica* cultivar, widely used in RS, has a higher sensitivity to salinity when compared to that of the *japonica* cultivar in other physiological aspects. Current research analyzes enzymes expression involved in salt-subjected *indica* and *japonica* rice cultivars' respiration. *Oryza sativa* L. spp. *japonica* S.Kato (BRS Bojuru, IAS 12-9 Formosa and Goyakuman) and *Oryza sativa* L. spp. *indica* S. Kato (BRS Taim-7, BRS Atalanta and BRS Querencia) were the cultivars employed. Seedlings were transferred to 15 L basins containing 50% Hoagland nutrient solution increased by 0, 25, 50, 75 and 100 mM NaCl, and collected at 14, 28 and 42 days after transfer (DAT). Plant tissues were macerated and placed in *eppendorf* tubes with Scandálios extractor solution. Electrophoresis was performed in 7% of the polyacrylamide gels in vertical vats. Bands were revealed for the following enzymes systems: esterase, alcohol dehydrogenase, phosphoglucoisomerase, malate dehydrogenase, malic enzyme and alpha amylase. The enzymes expression was greater in subspecies *japonica,* with more intense bands in proportion to salinity increase. Results show that enzyme systems are involved in the salinity defense mechanisms in *O. sativa* spp. *japonica* cultivar.

Keywords: electrophoresis, isoenzyme, krebs cycle, *O. sativa* L., salinity.

Expressão de enzimas em cultivares de arroz *indica* e *japonica* sob estresse salino

RESUMO. O Estado do Rio Grande do Sul (RS) destaca-se como principal produtor de arroz, participando com 60% da produção nacional e 86% da regional**.** O sistema de irrigação da cultura é por inundação, que induz o solo à salinização, um dos maiores limitadores ambientais à produção, alterando o metabolismo da plantas expostas a este tipo de estresse. As cultivares *indicas* amplamente utilizadas no RS demonstram maior suscetibilidade à salinidade quando comparadas às *japonicas* em outros aspectos fisiológicos. O objetivo da pesquisa foi analisar a expressão de enzimas envolvidas na respiração de cultivares de arroz, *indica* e *japonica*, submetidas à salinidade. Foram utilizadas cultivares de *Oryza sativa* L. spp. *japonica* S. Kato (BRS Bojuru, IAS 12-9 Formosa e Goyakuman) e de *Oryza sativa* L. spp. *indica* S. Kato (BRS-7 Taim, BRS Querência e BRS Atalanta). As plântulas foram transferidas para bacias de 15 L, contendo solução nutritiva de Hoagland meia força acrescida de 0, 25, 50, 75 e 100 mM de NaCl. A coleta foi aos 14, 28 e 42 dias. Os tecidos vegetais foram macerados e colocados em tubos *eppendorf* com solução extratora de Scandálios. A eletroforese foi realizada em géis de poliacrilamida 7% em cubas eletroforéticas verticais. As bandas foram reveladas para os sistemas enzimáticos esterase, álcool desidrogenase, fosfoglico isomerase, malato desidrogenase, enzima málica e alfa amilase. A expressão das enzimas foi maior na subespécie *japonica,* com bandas mais intensas conforme o aumento da salinidade. Conclui-se que tais sistemas enzimáticos estejam envolvidos em mecanismos de tolerância ao estresse salino nas cultivares de *O. sativa* spp. *japonica.*

Palavras-chave: eletroforese, isoenzimas, ciclo de krebs, *O. sativa* L., salinidade.

Introduction

Although total affected area by salinity in Brazil is 2% (MENEZES-BENAVENTE et al., 2004), it has become a major obstacle to crop productivity (DASGAN et al., 2002), especially in the southern State of Rio Grande do Sul, Brazil, where *Oryza sativa* L. culture is irrigated by flooding. Saline water comes from the Laguna dos Patos and coastal rivers which are subjected to salinization due to the introduction of sea water when water levels are low (MARCOLIN et al., 2005). This fact mainly occurs during the summer

when low rainfall and high evaporation-transpiration coincide with the culture's reproduction phase.

Rice is moderately tolerant to salinity. However, the widespread use of *indica* rice cultivars in RS may be one of the most impairing factors in the process, since *japonica* cultivars have a higher salt tolerance than that of *indica* cultivars (LIMA et al., 2004). The plant needs osmosis adjustments to adapt itself to stress which involves increased energy expenditure due to the synthesis of organic solutes (FLOWERS et al., 2010). The latter act as enzymatic protection (GREENWAY; MUNNS, 1980) and cause disturbances in the general metabolic activity (MANSOUR; SALAMA, 2004), including photosynthesis (WILLADINO et al., 2011), enzymatic activity (MENEZES-BENAVENTE et al., 2004; SOUZA FILHO et al., 2003) and respiration (TAIZ; ZEIGER, 2004). Thus, enzymes involved in respiration may somehow provide a carbon source for regulating ionic and osmotic adjustment (TAIZ; ZEIGER, 2004), synthesis of new molecules) and removal of toxic substances during anaerobic respiration (FARIA et al., 2003). Moreover, they have an important role in Krebs cycle through photorespiration (MANCHENKO, 2000).

Isozyme analysis is employed to characterize rice cultivars (BONOW et al., 2001) and mobility differences in an electric field are the result of differences at DNA sequence levels that encode the enzymes. When band patterns of two individuals differ, it is presumed that that these differences have a genetic basis (MURPHY et al., 1990). The presence or absence of bands and changes in their intensity significantly differentiate the plants' isozymes (ORASMO; MACHADO, 2003). In fact, the environment is an important factor in the modulation of gene expression. The monitoring of such changes may be done by molecular markers. They not only provide useful data on the structure and genetic diversity of plant populations, but also visualize enzyme activity at the plant's different stages (ALFENAS, 1991).

It is therefore highly important that farmers understand the mechanisms of adaptation and acclimation to salt stress through a combined biochemical and physiological research and thus, the development and use of tolerant cultivars (SHINOZAKI; DENNIS, 2003), coupled to the reuse of degraded areas and the employment of low quality water.

Current research evaluates the respiration enzymes expression in *Oryza sativa* spp. *japonica* S. Kato and *Oryza sativa* spp. *indica* S. Kato cultivars under salt stress.

Material and methods

Subspecies of *Oryza sativa* spp. *japonica*, tolerant to salinity, and *Oryza sativa* spp. *indica*, susceptible to salt stress, were used in the experiment (LIMA et al., 2004, 2005). Whereas cultivars of *Oryza sativa* L. spp. *japonica S*. Kato comprised BRS Bojuru, IAS 12-9 Formosa and Goyakuman, cultivars of *Oryza sativa* L. spp. *indica* S. Kato comprised BRS-7 Taim, BRS Querência and BRS Atalanta.

The seedlings were obtained by sowing in plastic trays containing washed sand as a substrate. Ten days after emergency (DAE) seedlings were transferred to a greenhouse with controlled temperature and humidity, and placed in 15L-basins containing 12 L Hoagland nutrient solution (HOAGLAND; ARNON, 1950), 50%, with concentrations 0, 25, 50, 75 and 100 mM NaCl, based on previous research with the same cultivars, and changed at a 4-day interval. Styrofoam plates were used to prop the 30 seedlings fixed with cotton. They were kept in the basins for 42 days after transfer (DAT). During this period 3 sample collections were undertaken every 14 days. The plants were harvested at 14, 28 and 42 DAT, packed in identified plastic bags, with wet cotton, and placed in styrofoam boxes with ice for transport to the laboratory. They were immediately stored at -70°C in an ultrafreezer until further analysis.

The tests were conducted in the BioSeeds Laboratory, Department of Phytotechnology of the Eliseu Maciel Faculty of Agronomy, Federal University of Pelotas, Pelotas, State of Rio Grande do Sul, Brazil. Plant tissues (the last three leaves from the base to the apex) were ground in a porcelain mortar with ice. Aliquots ranging between 200 and 300 mg of the plant extract were placed in an *eppendorf* tube with an extractor gel buffer solution (Tris 0.051 M, 0.0076 M citric acid and water qsp 1 L), electrode buffer (Lithium hydroxide 0.38 M, 0.192 M boric acid and water qsp 1 L) and 0.15% of 2-mercaptoethanol at 9:1:2 (w v^{-1}) (SCANDÁLIOS, 1969). They were then stored in a refrigerator for 24 hours for enzyme extraction after centrifuged at 16,100 g for 5 min., at 4°C. Electrophoresis was undertaken in 7% polyacrylamide gels by placing 20 μL of each sample in acrylic comb-produced wells. The enzymatic patterns were analyzed by the buffer system (SCANDÁLIOS, 1969). Gels were placed in vertical electrophoretic vats and kept in a cold chamber at 4-6°C. The electrophoretic migrations were undertaken with a potential difference of

1.0 V mm^{-1}, until the front line formed by bromophenol blue reached 90 mm from the point of application.

Bands were revealed for the enzyme systems esterase (EST), according to Scandálios (1969), alcohol dehydrogenase (ADH), phosphoglucose isomerase (PGI), malate dehydrogenase (MDH) and malic enzyme (ME), described by Alfenas (1991), and alpha amylase (α - AMY), according to Alfenas (1998). Electrophoresis gels were fixed in the solution of distilled water: methanol: acetic acid at 5:5:1 (v v^{-1}). Taking into account the presence / absence and intensity of each electrophoretic band, results' interpretation was based on visual analysis of electrophoresis gels.

Results and discussion

Although esterase electrophoretic patterns (EST - EC 3.1.1.1) revealed the presence of the enzyme in the three tillage periods (14, 28 and 42 DAT), highest intensity of bands was reported at 28 DAT. In first harvest, at 14 DAT, the *japonica* cultivars Bojuru (B); Formosa (F) and GoyaKuman (G), tolerant to salinity, and the *indica* cultivar Taim (T), susceptible to salinity, had a higher esterase expression. Bands' intensity among the tolerant Formosa cultivars increased in salinity accordingly. This fact suggested higher enzyme activity when subjected to salt stress. The same pattern was obtained for all *japonica* varieties tested at 28 DAT (Figure 1).

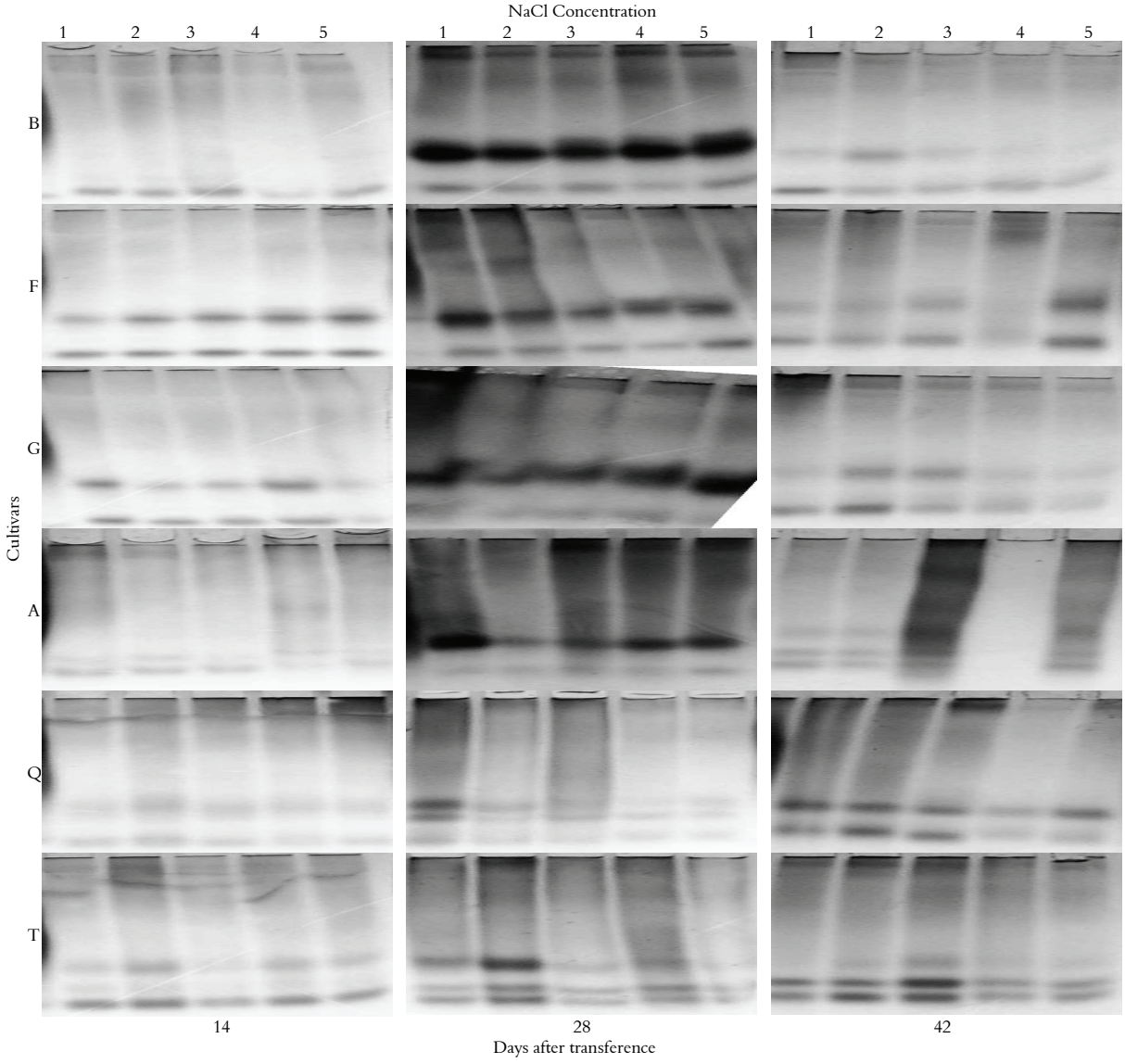

Figure 1. Electrophoretic patterns obtained with esterase (EST) enzyme in rice leaves on cultivars Bojuru (B), Formosa (F), GoyaKuman (G), Atalanta (A), Querência (Q) and Taim (T), at 14, 28 and 42 days after transfer to solutions 0 (1), 25 (2), 50 (3), 75 (4) and 100 (5) mM NaCl.

With the exception of differential expressions between tolerant and susceptible genotypes, no variation in response intensity to different salt concentrations occurred. In the third collection, the bands' intensity decreased in all cultivars when compared to that of previous periods. However, bands in Querência and Taim cultivars were more intense up to 50 mM NaCl treatment. Cultivar Formosa showed a more intense band with higher salt concentrations (100 mM) and may indicate the involvement of esterase in the cultivar's possible tolerance to salt stress (Figure 1).

The change in enzyme expression in proportion to salinity level is possibly related to some mechanism of plant defense. In fact, esterase is involved in lipid catabolism. Such degradation may be a carbon source for the synthesis of new molecules (FLOWERS et al., 2010) which would justify a differential standard in the *japonica* Formosa cultivar. When Lima et al. (2004) researched the effect of salt stress on rice seedlings, they reported higher salinity tolerance in *O. sativa* spp. *japonica* cultivars. Environmental factors affect plants' metabolism, influence the activity of isoenzymes, especially esterase, peroxidases, phosphatases and phenolases, and cause different enzyme patterns or activities (MALONE et al., 2006).

Alcohol dehydrogenase (ADH - EC 1.1.1.1) showed activity only in the first collection, at 14 DAT (Figure 2), in all *japonica* cultivars and in the Taim *indica* cultivar. The presence of more highlighted bands in the *japonica* Bojuru cultivar than in other genotypes has been reported. In fact, there was an increase in their intensity in proportion to increase in NaCl concentrations.

ADH is an enzyme that acts on the respiratory process by removing toxic substances, such as acetaldehyde and ethanol, produced when cells start anaerobic respiration (FARIA et al., 2003). Therefore, when the aerobics path is impaired, the anaerobic respiration route is activated and cells' toxic substances, such as acetaldehyde and ethanol, are produced. In anaerobic metabolism (alcohol fermentation), pyruvate, primarily produced in glycolysis, is transformed into acetaldehyde by pyruvate decarboxylase enzyme and the acetaldehyde is reduced to ethanol by ADH. Plants with increased resistance to salinity compartimentize salt to reduce its harmful effects (FLOWERS et al., 2010). Compartmentalization may have occurred in cytosol and not in vacuoles of leaves of more resistant cultivars.

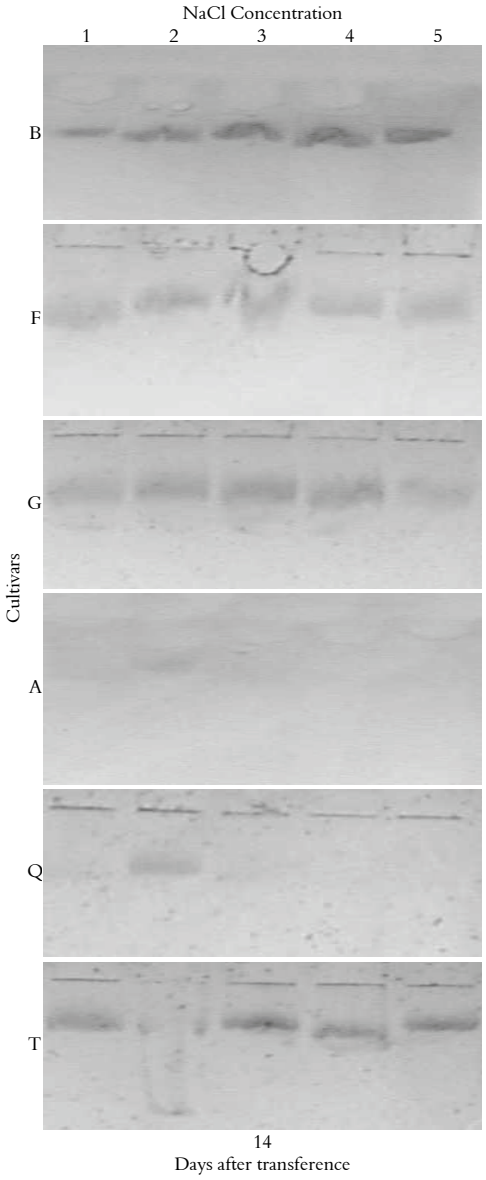

Figure 2. Electrophoretic patterns of alcohol dehydrogenase (ADH) enzyme in rice leaves of cultivars Bojuru (B), Formosa (F), GoyaKuman (G), Atalanta (A), Querência (Q) and Taim (T), at 14, 28 and 42 days after transfer to solutions 0 (1), 25 (2), 50 (3), 75 (4) and 100 (5) mM NaCl.

This fact possibly triggered the fermentation process. ADH activation for ethanol production occurred to minimize salt damage to the cell and the detrimental effect of acetaldehyde accumulation. Evidence exists that acetaldehyde accumulation may be more detrimental to cell metabolism that ethanol accumulation (DREW, 1997). Moreover, since lipids are soluble in ethanol, their diffusion to the external environment through the membrane in which it would be diluted or metabolized by microorganisms, is possible.

Malate dehydrogenase (MDH - EC 1.1.1.37) zymograms showed enzyme activity in the three collection periods even though greater intensity of

MDH has been verified at first sampling (14 DAT) in all cultivars. Among the *japonica* cultivars (Figure 3B, F and G), Bojuru had more intense bands in the three collections and in the more saline treatments. Cultivars Formosa and GoyaKuman showed a similar enzymatic pattern in the first and second collections. The bands in the GoyaKuman cultivar were more intense according to increasing NaCl concentrations. *Indica* cultivars (Figure 3A, Q and T) showed similar expression pattern among themselves in three tillages. However, the Atalanta cultivar had more intense bands in the three lower doses in the first collection and virtually no activity in the third one. It is an indication of higher susceptibility to increased saline concentrations.

Enzyme MDH occurs in the mitochondrial matrix and in cell cytoplasm and functions in the mitochondrial during the two final reactions of the citric acid cycle. NADH is thus oxidized in the mitochondrial respiratory chain to produce ATP by oxidative phosphorylation (TAIZ; ZEIGER, 2004). In the cytoplasm, MDH catalyzes the reaction of OAA reduction, producing NAD+ required for glycolysis, and may also oxidize mitochondria-transported malate, to produce sucrose by glyconeogenic pathway. MDH has an important role in Krebs cycle (MANCHENKO, 2000) since it participates in the malate movement through the mitochondrial membrane and CO_2 fixation. Further, it also has a metabolic function in NADPH production, in photorespiration, ionic regulation and osmotic adjustment (TAIZ; ZEIGER, 2004).

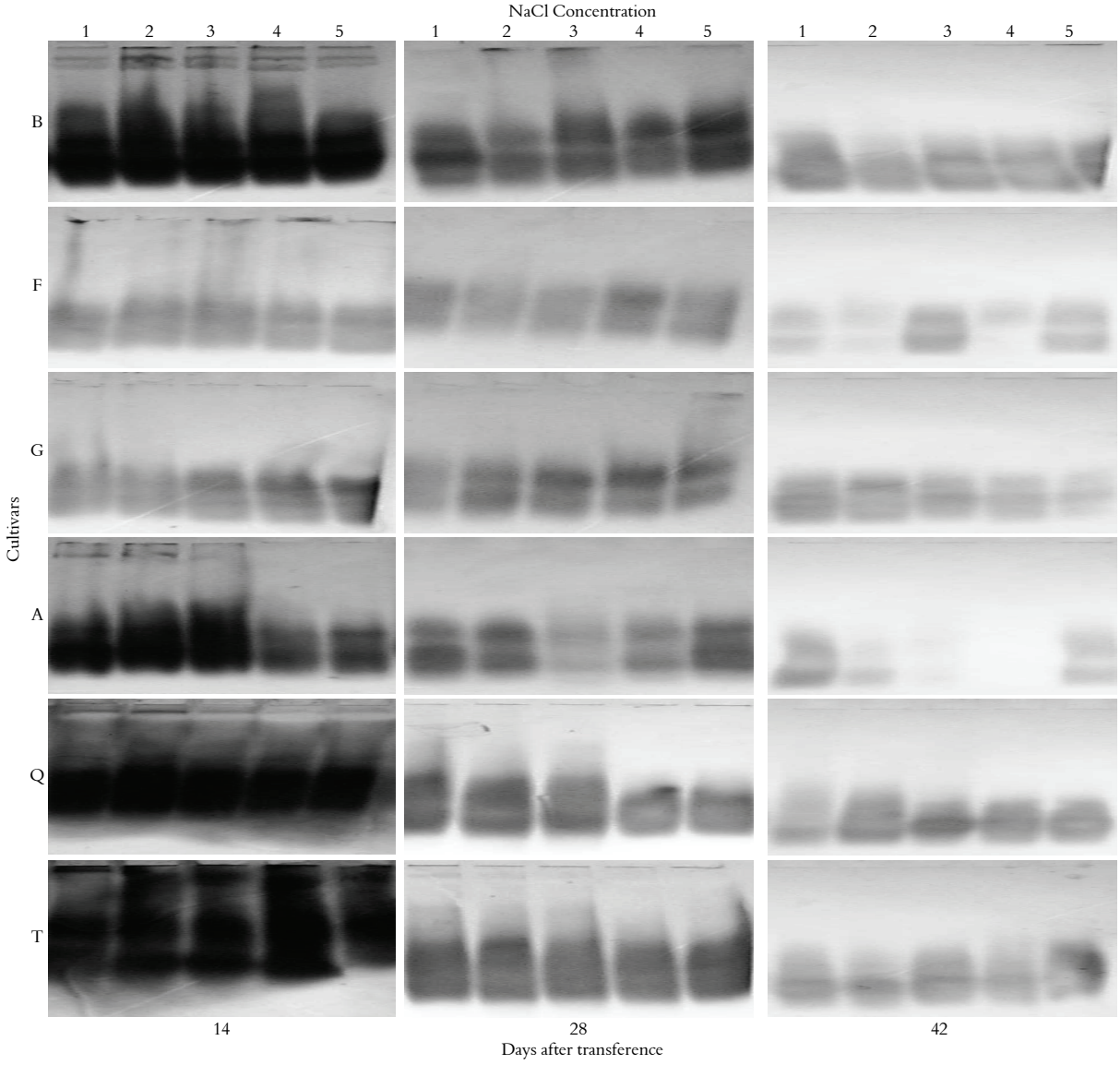

Figure 3. Electrophoretic patterns of malate dehydrogenase (MDH) enzyme in rice leaves on cultivars Bojuru (B), Formosa (F), GoyaKuman (G), Atalanta (A), Querência (Q) and Taim (T) at 14, 28 and 42 days after transfer to solutions of 0 (1), 25 (2), 50 (3), 75 (4) and 100 (5) mM NaCl.

Since it is a constitutive enzyme, MDH is important because it shows activity in all evaluated cultivars and sampling periods. Plants under salt stress make osmotic adjustments by the accumulation of ions in the vacuole and by the synthesis of compatible solutes in the cytosol, such as glycine-betaine, sorbitol, mannitol, sucrose and proline (SZABADOS; SAVOURÉ, 2009). They all have the protective function of the plasma membrane (MANSOUR; SALAMA, 2004) and the cytosol enzymes. The amount of carbon used for the synthesis of organic solutes is high and carbon diversion for the adjustment of water potential reduces growth, total biomass and crop productivity. MDH pattern suggests increased activity of the enzyme in cultivars when the environment's salinity increases. Tolerant rice cultivars have an increased MDH activity as salinity increases (KUMAR et al., 2000). These data corroborate the findings of current research.

The Atalanta cultivar showed phosphoglicoisomerase expression (PGI - EC 5.3.1.9) only in the first sampling of treatments 0, 25 and 50 mM NaCl (Figure 4).

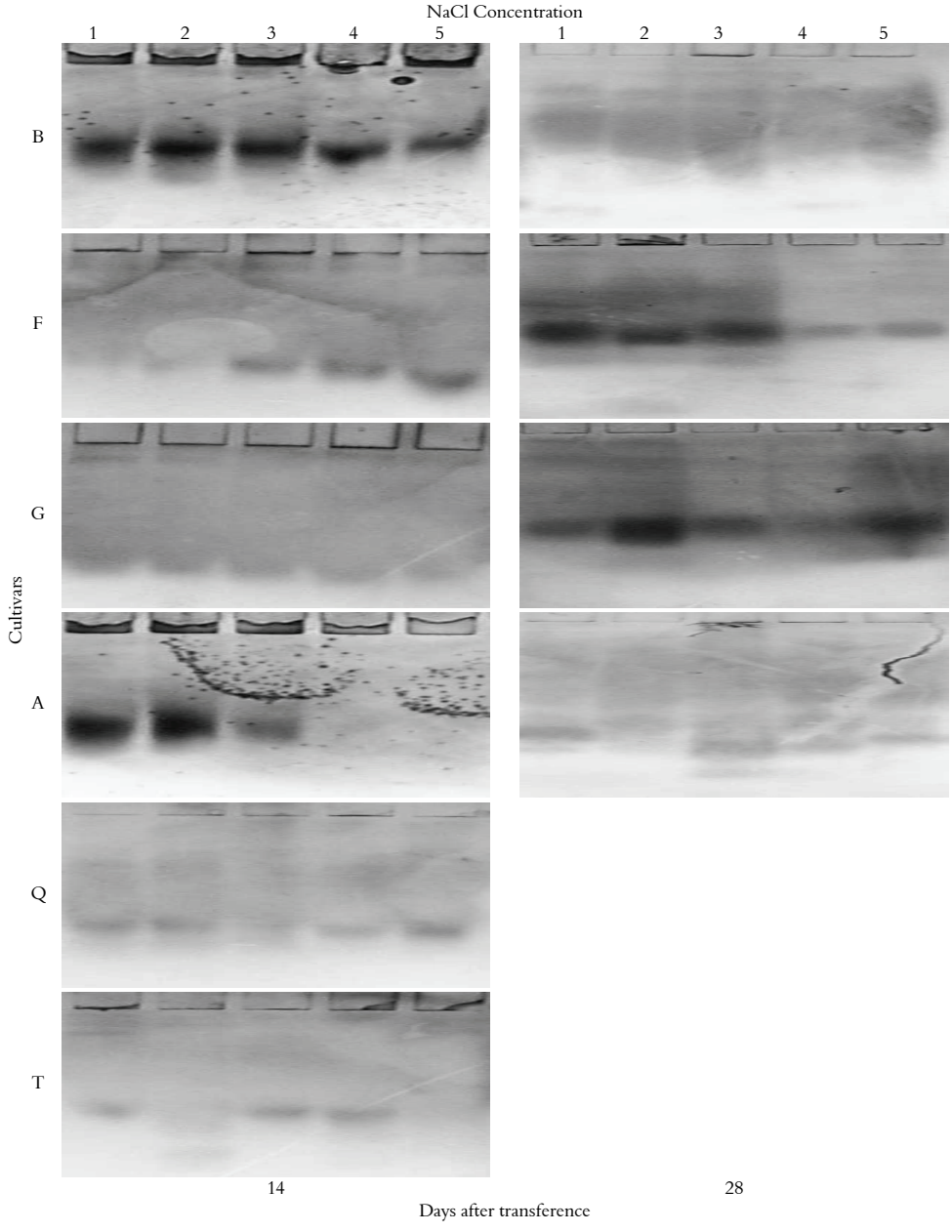

Figure 4. Electrophoretic patterns of phosphoglicoisomerase (PGI) enzyme in rice leaves on cultivars Bojuru (B), Formosa (F), GoyaKuman (G), Atalanta (A), Querência (Q) and Taim (T), at 14, 28 and 42 days after transfer to solutions of 0 (1), 25 (2), 50 (3), 75 (4) and 100 (5) mM NaCl.

While Bojuru cultivar revealed bands with similar intensity patterns for all treatments, bands in the Formosa cultivar were detected during the first collection at concentrations 50, 75 and 100 mM NaCl and at concentrations of 0, 25 and 50 mM in the second collection. Bands in GoyaKuman cultivar were more intense in the second collection and at salinity levels 25 and 100 mM.

Operating in glycolysis, PGI enzyme catalyzes the reversible isomerization from G6P to F6P (MANCHENKO, 2000; TAIZ; ZEIGER, 2004). Consequently, its presence in *japonica* cultivars may indicate an alternative mechanism to compensate the demand for carbon skeletons for the production of organic solutes that act in osmotic adjustment. According to Greenway and Munns (1980) and others, it is a strategy employed by salinity-tolerant species.

The α-amylase (α-AMY - EC 3.2.1.1) enzyme was not detected in all cultivars, concentrations and stages under analysis (unpublished data). Cultivars have probably shown enzyme expression in a period previous to plants' exposure to salinity. This is due to the fact that the enzyme causes starch hydrolysis during the germination process and early growth, which may justify the non-formation of bands. Highest enzyme activity in seeds occurs approximately ten days after germination. Within the initial phase, growth is slow and photo-assimilates demand is high in roots and new leaves growth. Starch accumulation that would favor α-amylase synthesis fails to occur (BEWLEY; BLACK, 1994).

Malic enzyme (EM - EC 1.1.1.40) zymograms indicate that it had activities during the three sampling periods, or rather, 14, 28 and 42 DAT (Figure 5).

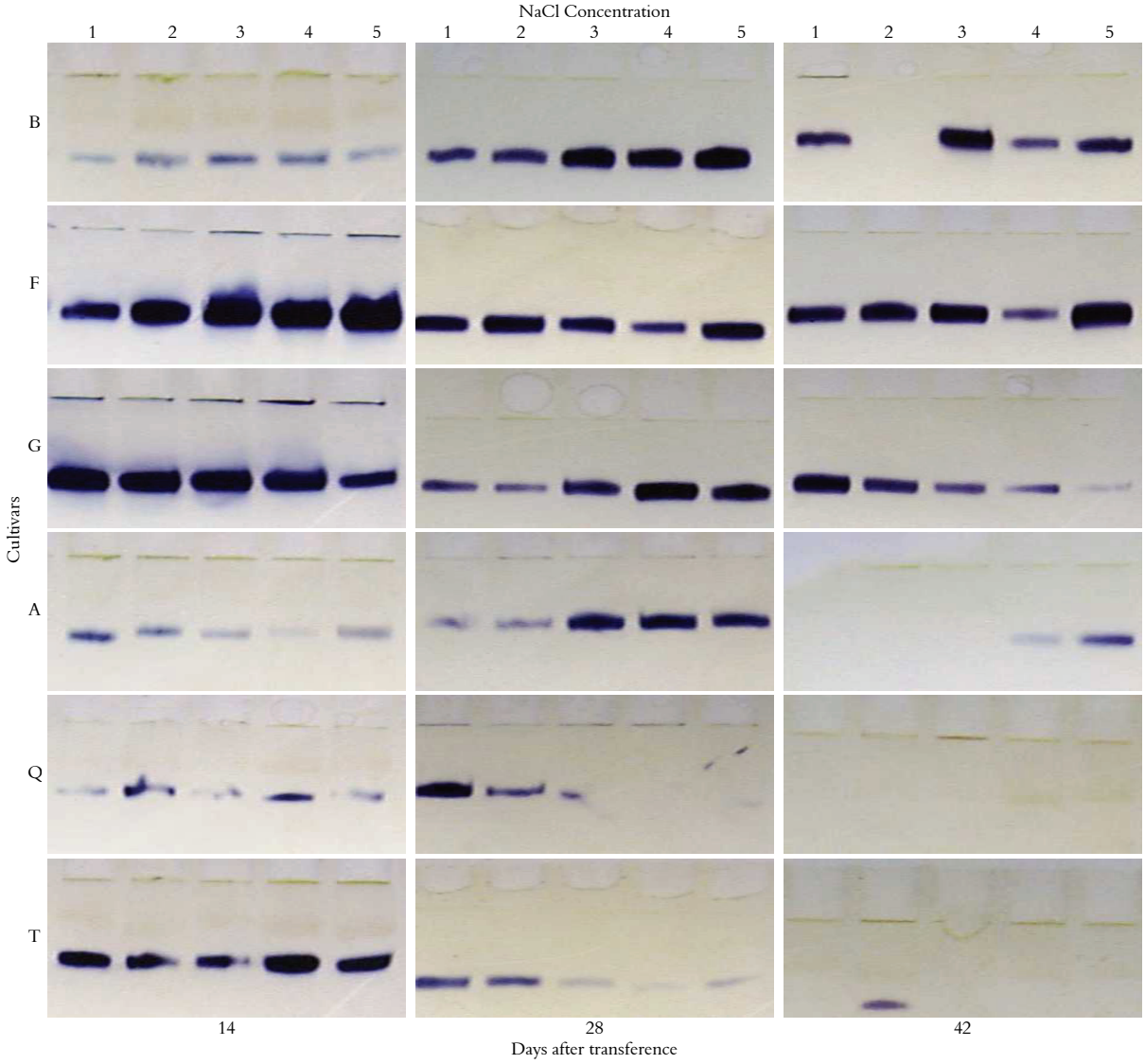

Figure 5. Electrophoretic patterns of malic enzyme (ME) in rice leaves in cultivars Bojuru (B), Formosa (F), GoyaKuman (G), Atalanta (A), Querência (Q) and Taim (T), at 14, 28 and 42 days after transfer to solutions 0 (1), 25 (2), 50 (3), 75 (4) and 100 (5) mM NaCl.

Bands of enzyme activities in the first collection were greater than those in *indica* Taim cultivar and in *japonica* GoyaKuman and Formosa cultivars. The latter had more intense bands according to increasing salinity. Bands in the second collection showed a higher intensity in *japonica* Bojuru and GoyaKuman cultivars at concentrations 50, 75 and 100 mM NaCl. As a rule, Formosa had similar activity in the different treatments. Within the context of *indica* cultivars, Atalanta exhibited bands at higher concentrations, although the others showed activity in control and in 25 mM NaCl. Only the *japonica* Bojuru, Formosa and GoyaKuman cultivars expressed any activity in the third collection, whereas bands in the Formosa cultivar showed uniformity in intensity either in control or in the other treatments.

A band in the Atalanta cultivar was detected at the highest salt concentration although with less intensity than that obtained at 28 DAT. Moreover, in the GoyaKuman cultivar, enzyme expression decreased with increasing salinity. Malic enzyme decarboxylates malate into pyruvate with mitochondria operating alternative pathways for the metabolism of glycolysis-derived phosphoenolpyruvate (PEP) and providing metabolic flexibility to plants (TAIZ; ZIEGER, 2004). This mechanism suggests that plants undergoing salinity transform pyruvate into ethanol under anaerobic conditions, as mentioned earlier (Figure 3). In its absence or lack, the plants oxidize malate or citrate in alternative routes without the involvement of glycolysis-derived pyruvate. Consequently, flexibility is provided to saline-stressed plants as a response to such environment.

Current research was undertaken to identify which rice genotypes belonging to the subspecies *indica* and *japonica* were susceptible and tolerant to salinity. In the first place, there was a positive response of the subspecies *japonica* cultivars. The information triggered the search for adaptation mechanisms of the two subspecies and how they responded to salt stress. This search became the focus of current study. The evaluation of certain enzymes' expression involved in respiration becomes interesting since respiration is the source of energy for plants to support growth and development. These factors comprise the overcoming of difficulties in the environment, often due to anthropogenic issues brought about by the misuse of natural resources.

Conclusion

Although some are constitutive enzymes, results may imply that the enzymatic systems ADH, MDH, ME and PGI at 14 DAT, and EST and ME at 28 DAT are more expressed and that *japonica* cultivars have more intense bands in proportion to increasing salinity. Evaluated enzyme expression suggests that the above-mentioned cultivars resort to these mechanisms to tolerate stress. In fact, they are employed as an alternative route for metabolism, carbon and energy source for the synthesis of new molecules, in toxic substances removal and in ionic regulation and/or osmotic adjustment.

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