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Riboflavin Induces Different Defense Responses against *Pyricularia oryzae* in Improved and Traditional Rice (*Oryza sativa* L.) Cultivars

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ABSTRACT

Riboflavin (vitamin B_2) affects plant growth and development and participates in a variety of redox processes that affect plant defense responses. Two rice cultivars Fajr (improved) and Tarom Mahali (traditional) were foliar sprayed with increasing concentrations of riboflavin (0, 0.5, 1, 1.5 and 2 mM) and subsequently infected by *Pyricularia oryzae.* Then, leaves were collected at 0, 2, 4, 6 and 8 days after infection and activity of Peroxidase (POD), PolyPhenol Oxidase (PPO), and Phenylalanine Ammonia Lyase (PAL) were measured. Results revealed that lesion size and percentage of infected rice plants in Fajr was higher than Tarom Mahali. In addition, riboflavin-induced resistance was higher in Fajr than in Tarom Mahali due to higher activity of POD, PPO and PAL in Fajr than Tarom Mahali, especially upon exposure of plant to 2 mM riboflavin. The intensity of the bands of peroxidase isoenzymes with low molecular weight was enhanced by increasing concentrations of riboflavin in both rice cultivars, while elevated riboflavin concentration caused the synthesis of three new isoenzymes in Fajr (g, h, i) and one (f) in Tarom Mahali cultivars. It can be concluded that Fajr is more sensitive to infection of *P. oryzae* than Tarom Mahali. In addition, the activity of POD, PPO, and PAL enhanced intensity of peroxidase isoenzymes bands. Also, the synthesis of new isoenzymes by riboflavin showed that riboflavin-induced resistance was more effective in Fajr than in Tarom Mahali.

Keywords: Fajr cultivar, Peroxidase isoenzymes, Phenylalanine ammonia lyase, Polyphenol oxidase, Tarom Mahali cultivar.

INTRODUCTION

Rice blast is caused by *Pyricularia oryzae* and occurs in most rice growing areas all over the world. It is considered as one of the most important diseases of rice due to its worldwide distribution, destructiveness, and high degree of pathogenicity (Talbot and Foster, 2001; Thuan *et al*., 2006; Park *et al*., 2009).

Disease occurs only when the defense mechanisms of susceptible plants respond more slowly and/or weakly after pathogen attack and consequently the pathogen can escape from the plant defense. In contrast, resistance results from durable defense mechanisms leading to the absence of pathogen growth and subsequently to its death. The defense mechanisms include hypersensitive cell death, the production of Active Oxygen Species (AOS), cell wall modifications and accumulation of Pathogenesis Related (PR) proteins in plants (Norman-Setterblad *et al*., 2000; Faize *et al*.,

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2004; Glazebrook, 2005; Torres *et al*., 2006). Fungicides are helpful to maintain crop production by protecting plants against fungal diseases (Aguin *et al*., 2006). In recent years, there is an increasing interest in finding alternatives to fungicides that are considered as safe with negligible risk to human health and the environment (Boubakri *et al*., 2016).

An alternative to control *P. oryzae* is application of natural compounds that control blast disease by induction of resistance (Groth, 2006). Foliar application of riboflavin as a natural compound effectively induces defense response against pathogens, for instance, in sugar beet against *Rhizoctonia solani* (Taheri and Tarighi, 2011), chickpea against Fusarium wilt (Saikia *et al*., 2006), bean against *Botrytis cinerea* (Azami-Sardooei *et al*., 2010), grapevine against *Plasmopara viticola* (Boubakri *et al*., 2013) and rice against *P. oryzae* (Averyanov *et al*., 2000). In addition, effective control of pathogens was achieved by exogenous application of riboflavin in combination with methionine (Kang, 2008) or niacin (Pushpalatha *et al*., 2007). However, the mechanisms for defense activation by riboflavin are not well understood. Zhang *et al.* (2009) suggested that the role of riboflavin in priming defenses was in a signaling process distinctive from the known pathways of hormone signal transduction. However, Taheri and her colleague indicated that riboflavin induced resistance in *Oryza sativa* against *R. solani* via jasmonate-mediated priming of phenylpropanoid pathway (Taheri and Tarighi, 2010). Moreover, involvement of phospholipases C and D in inducing defense responses by riboflavin was reported in tobacco cells (Wang *et al*., 2013). Exogenous application of riboflavin induced defense-related enzymes, such as Peroxidase (POD) (Saikia *et al*., 2006; Kang, 2008; Abdel-Monaim, 2011), PolyPhenol Oxidase (PPO) (Kang, 2008; Abdel-Monaim, 2011), Phenylalanine Ammonia Lyase (PAL) (Saikia *et al*., 2006; Taheri and Tarighi, 2010, 2011) in several plant-pathogen systems. Chitinase (Saikia *et al*., 2006; Abdel-Monaim, 2011) and callose deposition (Zhang *et al*., 2009; Boubakri *et al*., 2013) were also increased in the pathogen-inoculated plants treated with riboflavin.

Although riboflavin-induced defense response has been reported in several plant-pathogen systems, it may not be a general rule. As reported by Azami-Sardooei *et al*. (2010), riboflavin is not able to control *Botrytis cinerea* on tomato.

Introduction of improved rice cultivars is an important strategy for increasing rice productivity. The studies showed that Fajr as an improved rice cultivar is characterized with high yielding grain, desirable shape of seed, and favorable baking quality. Likewise, breeding pathogen-resistance rice cultivars is an important and primary aim of rice improvement program. However, Fajr cultivar is rather sensitive to some pest and pathogens such as *chilo suppressalis* (Ghahari *et al*., 2009), *P. oryzae* (Mousanejad *et al*., 2010) and *Rhizoctonia solani* (Okhovvat, 1999; Padasht-Dehkaeia *et al*., 2013). The aim of the present study was to get more insight into the significance of riboflavin in induction of resistance to rice blast disease caused by *P. oryzae* in improved (Fajr) and traditional (Tarom Mahali) rice cultivars.

MATERIALS AND METHODS

Fungal and Plant Material

Seeds of rice (*Oryza sativa* L. cv. Fajr and Tarom Mahali) were germinated in vermiculite in a climate-controlled room with 12 hours of artificial lighting at 300±20 µmol m^{-2} s^{-1}, day and night temperatures of 27 and 22±2°C, and, relative humidity of 70-80%. Two-week-old rice seedlings were transplanted to 25% Hoagland nutrient solution and grown there for six weeks.

The plants were foliar sprayed with increasing concentrations of riboflavin (0, 0.5, 1, 1.5 and 2 mM) containing 0.01%

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Tween 80 as surfactant. Ten mL of riboflavin solution was applied per plant using a portable hand sprayer; all leaves were sprayed uniformly. Then, after 2 hours, the plants were infected by spraying the sporal suspension on the surface of leaves.

Sporal suspension was prepared from 10-day old culture of *P. oryzae* (*P. oryzae* was grown on potato dextrose agar at 30°C and maintained at 4°C) by using distilled water containing 0.01% Tween 80. Sporal suspension contained 5.6×10^4 spore mL^{-1}. The percentage of infected rice plants was recorded and the lengths of all lesions per leaf were measured 8 days after infection with *P. oryzae*. Four measurements in each treatment and 10 plants in each measurement were used. Leaves were collected at 0, 2, 4, 6 and 8 days after infection.

To identify the effect of riboflavin on growth of *P. oryzae*, mycelial blocks (0.6 cm in diameter) of *P. oryzae* were cultured on potato dextrose agar supplemented with 0, 0.5, 1, 1.5 and 2 mM riboflavin at 30°C for 7 days and the diameter of the fungal colony was measured. Six measurements were performed for each treatment.

Plant Extraction and Enzymes Assay

Fresh plant leaves samples were collected from five different treatments, immediately transferred into liquid nitrogen and kept at -80°C. Four measurements in each treatment and 10 plants in each measurement were used. Frozen leaves samples were homogenized in 100 mM KH_2PO_4/Na_2HPO_4 buffer solution, containing 2 mM of EDTA, 1% (m/V) PVP and 1 mM Phenyl-MethylSulfonyl Fluoride (PMSF) with pH 7.0 (2 g fresh weight per 3 mL) at 0°C and filtered through one layer of Miracloth. The filtered extract was centrifuged at 12,000×g at 4°C for 15 minutes. The soluble protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Peroxidase

Peroxidase (POD, EC.1.11.1.7) activity was determined according to the method of Lin and Kao (2001). The reaction mixture contained 50 µL of 20 mM guaiacol, 2.8 mL of 10 mM phosphate buffer (pH 7.0) and 0.1 mL plant extract. The reaction was initiated by the addition of 20 µL of 40 mM H_2O_2. Absorbance at 470 nm was recorded for 1 min using UV-visible spectrophotometer (Spectrum; Sp-2100UV). The POD activity was calculated by using an absorption coefficient (26.6 mM^{-1} cm^{-1} at 470 nm) for the tetraguaiacol. One unit of POD activity was defined as the amount of enzyme required for formation of 1 µmol of tetraguaiacol per min at room temperature.

Polyphenol Oxidase

PolyPhenol Oxidase (PPO, EC.1.10.3.1) activity was determined according to the method of Siriphanich and Kader (1985). One mL reaction mixture contained 20 µL plant extract and 960 µL of 10 mM phosphate buffer with pH 7.0. Each sample was aerated for 2 minutes in a small test tube followed by adding 20 µL of 1,000 mM catechol as the substrate. PPO activity was presented as the change in one unit of absorbance at 420 nm per min per mg per mL protein.

Phenylalanine Ammonia Lyase

Phenylalanine Ammonia Lyase (PAL, EC.4.3.1.5) activity was measured following the method of Dickerson *et al*. (1984). The assay mixture containing 100 µL of plant extract, 500 µL of 50 mM Tris HCl (pH 8.8), and 600 µL of 1 mM *L*-phenylalanine, was incubated for 60 minutes at room temperature, and then the reaction was arrested by adding 2N HCl. The assay mixture was extracted with 1.5 mL of toluene by vortexing for 30 seconds. Toluene was recovered after centrifuging at

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1,000 rpm for 5 minutes. The absorbance of the toluene phase containing *trans*-cinnamic acid was measured at 290 nm against the blank of toluene. Enzyme activity was expressed as nmol *trans*-cinnamic acid released per min per mg per mL protein.

Peroxidase Activity Staining

Equal amounts of protein from leaves were mixed with loading buffer and subjected to discontinuous poly acrylamide gel electrophoresis under non-denaturing and non-reducing conditions. Samples were loaded into gels. Then after electrophoresis, the gels were stained for peroxidase activity. Enzyme analysis experiments were repeated at least three times. Peroxidase activity was visualized by incubating the gels in 50 mM sodium acetate buffer pH 4.5, containing 2 mM benzidine and initiating the reaction by the addition of 3 mM H_2O_2 (Rao *et al*., 1997).

Statistical Analysis

All experiments were conducted in a completely randomized design with four replications. Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). A one-way analysis of variance (ANOVA) was performed and the treatment means were compared using Tukey's HSD all-pairwise comparisons at the *P*= 0.05 level as a post-hoc test (see figures).

RESULTS AND DISCUSSION

Previous reports have shown that riboflavin induces plant defense responses and resistance against pathogens (Taheri and Tarighi, 2011; Boubakri *et al*., 2013; Wang *et al*., 2013). Mechanisms for the disease resistance induced by certain chemicals include the direct inhibition of pathogen growth (Vicentini *et al*., 2002) and/or the induction of plant resistance to pathogen infection (Nakashita *et al*., 2003).

In the present study, the effect of riboflavin on the growth of *P. oryzae* was determined *in vitro* by growing the pathogens in media supplemented with riboflavin ranging from 0 to 2 mM. The colony diameters showed the pathogen grew similarly at all concentrations on agar plates (Table 1). Riboflavin does not likely inhibit the pathogen directly and it may induce the plant resistance to pathogen infection as previously reported by Dong and Beer (2000), riboflavin shows no effect against *Alternaria alternate* grown on potato dextrose agar. Likewise, Taheri and her colleague (2006) observed no microscopic and macroscopic cell death on rice when the plant leaves were sprayed with different concentrations of riboflavin (0.01, 0.1 and 2 mM) in absence of any pathogen (Taheri and Höfte, 2006).

The percentage of infected plants with *P. oryzae* pathogen showed that Fajr cultivar (improved cultivar) was totally infected while Tarom Mahali (traditional cultivar) was 40% infected *(*Figure 1). The effect of riboflavin on plants infected by *P. oryzae* pathogen resulted in decrease in percentage in both Fajr and Tarom Mahali (Figure 1). In Fajr cultivar the number of infected plants treated with riboflavin was hardly changed by 1 mM, however, it was severely reduced by almost 60 % from 1.5 to 2 mM

Table 1. Effect of riboflavin at different concentrations on growth of *P. oryzae*.

Riboflavin (mM)	Colony diameter (cm)
0	3.93±0.5a
0.5	3.83±0.08a
1	3.94±0.11a
1.5	4.07±0.12a
2	3.9±0.25a

Data on growth of *P. oryzae* represent the mean of 6 measurements (±SD). Different letters indicate significant difference between treatments (P< 0.05; One-way ANOVA, Tukey's HSD all-pairwise comparisons as a post-hoc test).

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riboflavin. The percentage of infected plants in Tarom Mahali was moderately reduced from 1 to 2 mM riboflavin (Figure 1).

The lesion lengths in Fajr infected by *P. oryzae* were almost 2-fold higher than in Tarom Mahali (Figure 1). The lesion length was hardly changed in both infected cultivars by 0.5 mM riboflavin, while it was reduced with increasing concentrations of riboflavin from 1.5 to 2 mM (Figure 1). Altogether, it seems that Fajr cultivar is more sensitive to *P. oryzae* than Tarom Mahali. In addition, riboflavin was more effective in induction of resistance in Fajr cultivar in comparison to Tarom Mahali cultivar.

Plant defense enzymes are generally induced when plants are invaded by microorganisms or damaged by mechanical injuries. The defense related enzymes are accumulated in plants with the onset of induced systematic resistance. Treatment with various biotic and abiotic inducers leads to increases in activity of defense related enzymes such as POD, PAL and PPO. However, the extent and course of the increase vary according to the inducer and host plant (Madhaiyan *et al*., 2004).

The activity of PAL, PPO and POD in both infected plants by *P. oryzae*, was steadily increased with increasing concentration of riboflavin from almost 0.5 to 2 mM. Furthermore, the highest activity of all three enzymes was observed 8 days after treatment with 2 mM riboflavin (Figure 2).

The result showed that the activity of PAL in Fajr was increased about 8-fold 8 days after exposure of plant to 2 mM riboflavin, while that of the Tarom Mahali was increased by almost 3-fold (Figure 2). PAL, one of the key enzymes in the phenylpropanoid and flavonoid pathways, increased in both incompatible and compatible interactions between plants and pathogens (Silva *et al*., 2004). A knockout mutant of rice PAL showed increased susceptibility to root-invaded *P. oryzae* which suggests a role of this pathway in disease-resistance to blast fungus (Cho and Lee, 2015). It is documented that *rice Phenylalanine Ammonia-Lyase* gene (OsPAL4) is also associated with broad spectrum disease resistance including rice blast (Tonnessen *et al*., 2015). Observed increment of PAL enzyme in this study apparently supports the host plant resistance against *P. oryzae*.

PPO activity was immediately increased 2.5-fold after exposure of Fajr to 2 mM riboflavin compared to untreated plants (Figure 2). But PPO activity in Tarom Mahali was hardly changed upon exposure to 2 mM riboflavin and remained the same as in untreated plants.

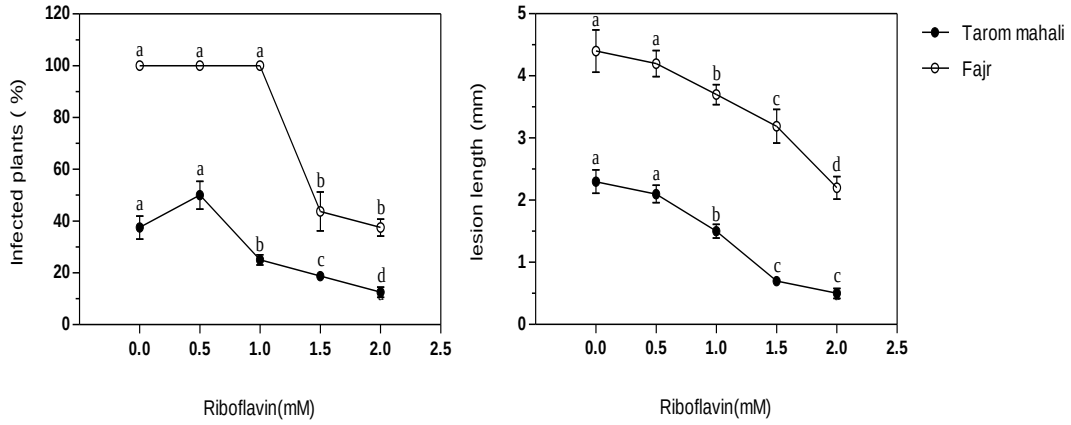

Figure 1. Effect of riboflavin at different concentrations on infected plants (%) and lesion size (mm) of the leaf of *Oryza sativa* L. cv. Fajr and Tarom Mahali infected with *P. oryzae*.

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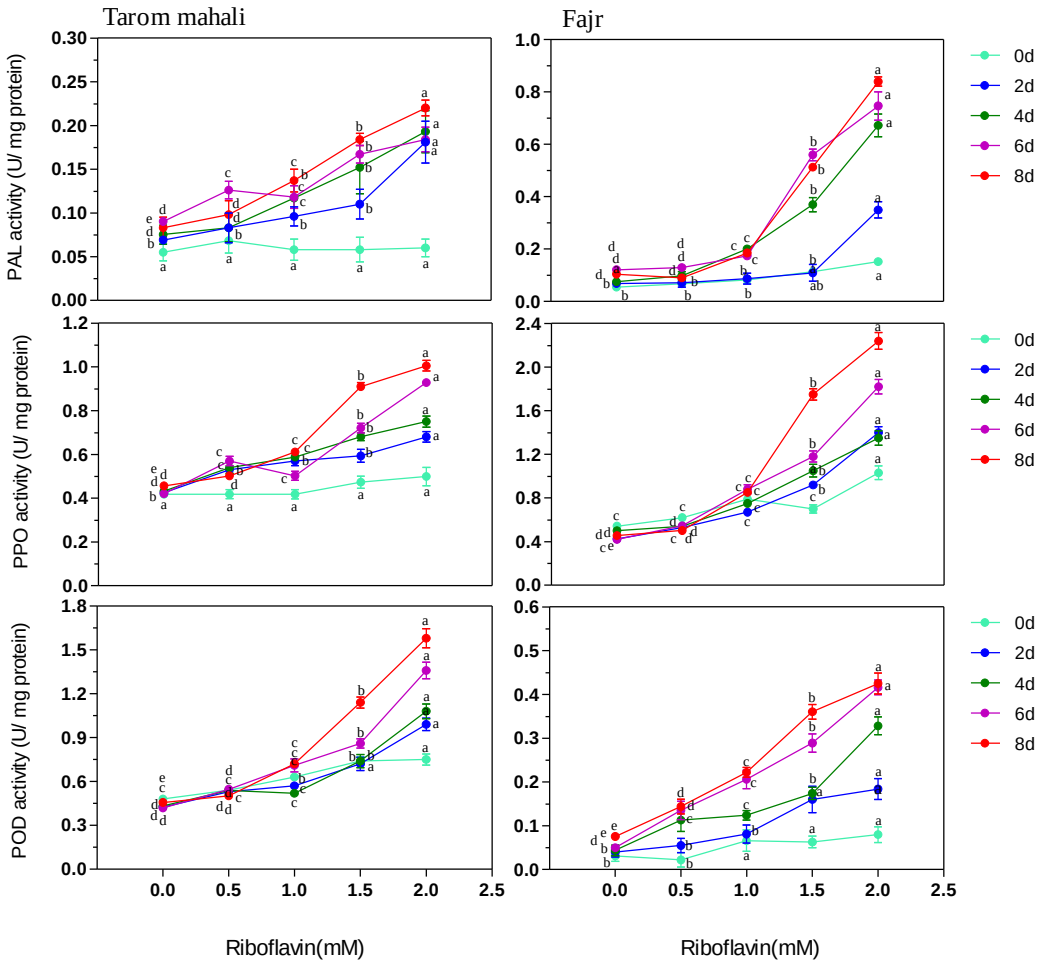

Figure 2. Effect of riboflavin at different concentrations on activity of POD, PPO and PAL in leaves of *Oryza sativa* L.cv. Fajr and Tarom Mahali infected with *P. oryzae*. Data on POD, PPO and PAL activity (U mg^{-1} protein) represent the mean of 4 measurements with 10 plants in each (±SD). Different letters indicate significant difference between treatments ($P< 0.05$; One-way ANOVA, Tukey's HSD all-pairwise comparisons as a post-hoc test).

The activity of PPO in both Fajr and Tarom Mahali was enhanced to the same level (around 2.5-fold) after 8 days exposure to 2 mM riboflavin (Figure 2). It is documented that PPO isoenzymes are also induced in both susceptible and blast resistant rice cultivars in response to *P. oryzae* (Malathi *et al*., 2014). Kang *et al*. (2008) suggested that the increase in lignin content with activation of PPO activity by foliar application of riboflavin and methionine accounted for the defense reaction to control the development of powdery mildew in cucumber. Mohammadi and Kazemi (2002) reported that PPO activity in resistant wheat cultivars were two times higher than sensitive cultivars when wheat heads were inoculated with *Fusarium graminearum.* Their result is in line with our findings and probably PPO is involved in lignification, and subsequently enhances plant defense against pathogen. PPO seems to be compartmentalized in plant cells, but as a result of pathogenic attack or during tissue senescence, membrane disruption may occur and this can probably initiate formation of quinones following an increase in

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accessibility of PPO to its substrate (Mohammadi and Kazemi, 2002).

POD activity was immediately increased 1.5-fold after exposure of both Fajr and Tarom Mahali to 2 mM riboflavin compared to that of the untreated plants (Figure 2). POD activity increased sharply after 6 and 8 days exposure to riboflavin from 1 to 2 mM. In Fajr, POD activity increased almost 5.5 fold after 8 days exposure to 2 mM riboflavin, while it increased 2.5 fold in Tarom Mahali (Figure 2).

In different plant pathogen interactions, the induction of POD is associated with resistance (Hu-zhe *et al*., 2005; Nyochembeng *et al*., 2007; Malathi *et al*., 2014). POD may function in defense through production of antimicrobial quantities of Hydrogen peroxide (H_2O_2) as well as in cell wall lignification or cross-linking with cell wall proteins (Novo-Uzal *et al*., 2013). An increase in POD activity was observed following infection of pepper by *P. capsici* (Hu-zhe *et al*., 2005), rice and rice blast fungus interaction (Sasaki *et al*., 2004), cucumber-powdery mildew (Kang, 2008) and wheat- *Fusarium graminearum* pathosystems (Mohammadi and Kazemi, 2002) Likewise, riboflavin induces resistance in rice against Rhizoctonia sheath disease by upregulation of rice POD and formation of lignin as structural barrier (Taheri and Höfte, 2006) which is in line with our finding.

Isoenzyme banding patterns of POD showed the presence of six isoforms in the leaves sprayed with riboflavin in Tarom Mahali cultivar. In this cultivar, intensity of isoenzymes e and f were enhanced by increasing concentrations of riboflavin, whereas these bands were faint in the control plants. These isoforms seem to be induced following the treatments with riboflavin (Figure 3A). Activity staining for POD revealed the presence of nine isoforms in Fajr cultivar. Several isoforms (a, b, c, d, e, and f) were constitutively present in all treatments and the control. In treatments of 1, 1.5 and 2 mM riboflavin, three isoforms (g, h, i) indicated higher staining intensity relative to the control and 0.5 mM riboflavin (Figure 3B).

The induction of new isoenzymes and

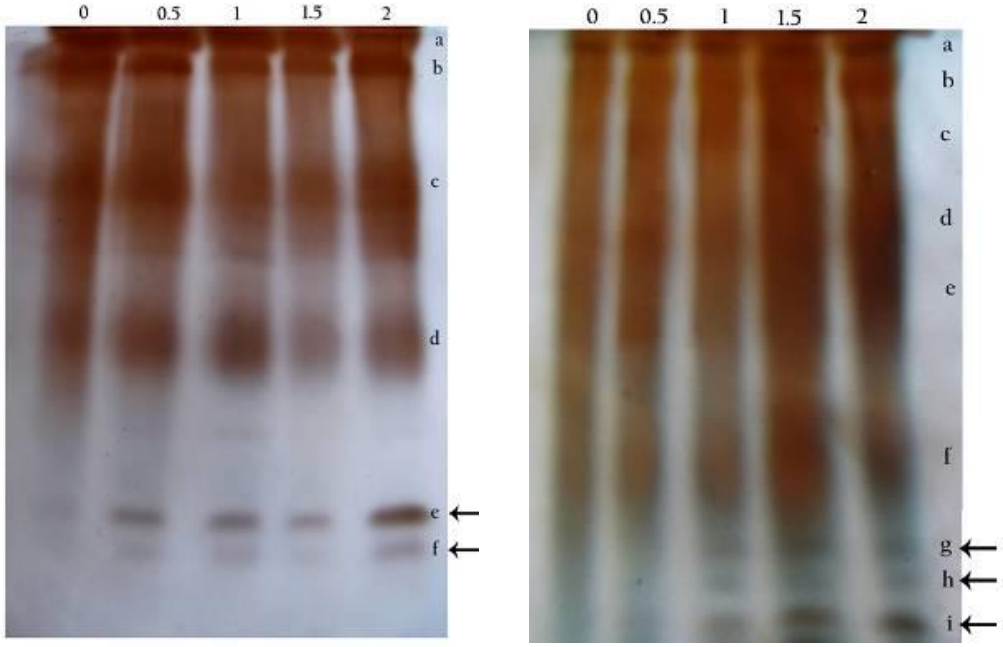

Figure 3. Effect of riboflavin at different concentrations on peroxidase isoforms in leaves of *Oryza sativa* L. cv. Fajr (B) and Tarom Mahali (A) infected with of *P. oryzae*. For experimental details see legends of Figure 1. Peroxidase isoforms separated by native PolyAcrylamide Gel Electrophoresis (PAGE). Different letters indicate different isoforms.

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increasing of isoenzymes intensity in rice cultivars confirm the importance of riboflavin in disease resistance response by POD enzyme. When riboflavin is exogenously applied by foliar spray onto *Arabidopsis thaliana*, perioxidation and antioxidative increased defense enzymes against *P. parasitica* and led to activation of PR genes. It suggests that riboflavin initiates the resistance signal transduction (Dong and Beer, 2000). The induction of POD isoenzymes was also observed in pepper roots after their infection by *P. capsici* zoospores (Hu-zhe *et al*., 2005). Many studies indicated that POD isoenzymes were induced by various abiotic and biotic inducers. In cucumber, POD isoenzymes in root were induced following pathogen challenge with *Pythium aphanidermatum* (Chen *et al*., 2000). Shivakumar *et al*. (2003) reported 22 POD isoenzymes in resistant pearl millet in comparison to 12 isoenzymes in susceptible plants. In our study, differential expression of POD isoenzymes in infected rice cultivars was distinguished at different concentrations of riboflavin. The relative intensity of the band e and the new appeared band f in Tarom Mahali cultivar could be correlated to induction of resistance against *P. oryzae*. Fajr cultivar exhibited three new isoenzymes g, h, and i that corresponded with riboflavin concentrations and were apparently the result of their preferential expression against *P. oryzae*. The results indicated that isoenzymes with higher molecular weight show little alterations at various concentrations of riboflavin in both cultivars whereas we observed the advent of POD isoenzymes with lower molecular weight. Furthermore, the most stained POD activity in these low molecular weight isoenzymes were observed in the treatment of 2 mM riboflavin. In general, the role of riboflavin in plant resistance against pathogens may derive from role of riboflavin as cofactor in enzymatic reactions. Indeed, flavoenzymes (FMN/FAD-dependent) are involved in a wide variety of biological processes such as redox processes, protein folding, nitrogen fixation, light sensing, chromatin remodeling, DNA repair and apoptosis (Miret and Munné-Bosch, 2014). Therefore, exogenous riboflavin probably induces or supports different known/unknown resistance responses against pathogens.

It can be concluded from the present study that Fajr was more sensitive to *P. oryzae* than Tarom Mahali. Likewise, riboflavin-induced resistance in Fajr was more perceived than Tarom Mahali. Riboflavin has a biological function in induction of the disease resistance against *P. oryzae* through increasing activity of enzymes such as PAL, PPO and POD in cells. It seems that POD isoenzymes with lower molecular weight are induced by riboflavin during resistance responses. Riboflavin may provide novel disease control strategies that satisfy environmental regulations.

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ریبوفلاوین پاسخ دفاعی علیه *Pyricularia oryzae* را به طور متفاوتی در ارقام اصلاح شده و محلی گیاه برنج القا می کند

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چکیده

ریبوفلاوین (ویتامین B_2) در رشد و نمو گیاهان و در بسیاری از فرایندهای احیایی که در پاسخهای دفاعی گیاهان نقش دارند، اثر گذارمی باشند. اندام هوایی دو رقم برنج اصلاح شده (فجر) و محلی (طارم محلی) با غلظت های افزایش یابنده ای از ریبوفلاوین (۰، ۱، ۱/۵، ۲ میلی مول) اسپری گردیدند و سپس در معرض آلودگی با قارچ *P. oryzae* قرار گرفتند. سپس اندام هوایی (برگها) در زمانهای مختلف صفر، ۲، ۴، ۶ و ۸ روز پس از آلودگی جمع آوری شدند و فعالیت آنزیم هایی از قبیل پراکسیداز، پلی فنل اکسیداز و فنیل آلانین آمونیا لیاز اندازه گیری شد. نتایج نشان داد، درصد گیاهان آلوده شده و اندازه بخشهای صدمه دیده برگها در رقم فجر بیشتر از طارم محلی بوده است. علاوه بر این، افزایش فعالیت آنزیم هایی از قبیل پراکسیداز، پلی فنل اکسیداز و فنیل آلانین آمونیا لیاز در رقم فجر نسبت به رقم محلی مخصوصا در غلظت ۲ میلی مول ریبوفلاوین نشان دهنده افزایش بالاتر مقاومت القا شده به وسیله ریبوفلاوین در رقم فجر نسبت به رقم طارم محلی می باشد. شدت باندهای ایزو آنزیمی پراکسیداز با وزن مولکولی پایین در هر دو رقم با افزایش غلظت ریبو فلاوین، افزایش یافته است در حالیکه غلظت بالای ریبو فلاوین منجر به تولید سه ایزوآنزیم در رقم فجر(g, h, i) و یک ایزوآنزیم در رقم طارم محلی(f) گردیده است. در نتیجه گیری کلی، رقم فجر به قارچ بیماری زای *P. oryzae* حساسیت بیشتری نسبت به رقم طارم محلی نشان داده است. علاوه بر این، فعالیت آنزیمهای PPO،POD و PAL، افزایش شدت باندهای ایزو آنزیمی پراکسیداز و تولید ایزوآنزیمهای جدید دراثرافزیش غلظت ریبو فلاوین، نشان دهنده افزایش بالاتر مقاومت القا شده به وسیله ریبوفلاوین در رقم فجر نسبت به رقم طارم محلی می باشد.

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