BIOLOGIA PLANTARUM 54 (2): 329-333, 2010

BRIEF COMMUNICATION

Stimulation of antioxidative enzymes and polyamines during stripe rust disease of wheat

B. ASTHIR[1]*, A. KOUNDAL[1], N.S. BAINS[2] and S.K. MANN[3]

Department of Biochemistry[1], Department of Plant Breeding and Genetics[2], and Department of Plant Pathology[3], Punjab Agricultural University, Ludhiana-141004, Punjab, India

Abstract

Content of polyamines and activities of antioxidative enzymes in response to stripe rust disease caused by *Puccinia striiformis* have been studied in two wheat (*Triticum aestivum* L.) cultivars PBW 343 (resistant) and HD 2329 (susceptible). Various infection stages ranging from traces to 100 % were collected. Infection leads to stimulation of peroxidase (POD), superoxide dismutase (SOD), catalase, diamine oxidase and polyamine oxidase activities along with increase in putrescine, spermidine and spermine content while ascorbate, tocopherol and chlorophyll content decreased in HD 2329 and no visible symptoms appeared in PBW 343. Histochemical localization pattern of POD and SOD activities revealed correlation with lignin deposition in cell walls.

Additional key words: diamine oxidase, peroxidase, polyamine oxidase, *Puccinia striiformis*, *Triticum aestivum.*

Stripe rust, also known as yellow rust, caused by *Puccinia striiformis* f. sp. *tritici*, is a widespread fungal disease hampering wheat production worldwide (Sache and de Vallavieille-Pope 1993). Consequently, major effort has been devoted to development of stripe rust resistant cultivars.

A common consequence of most biotic stress is an increased production of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) (Suzuki and Mittler 2006). Excessive levels of ROS is potentially damaging to plant cells unless detoxified by antioxidative system (Agarwal *et al*. 2005). Peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) are considered to be the main protective enzymes, being engaged in the removal of free radicals and ROS (Liu *et al*. 2008). SOD is the key enzyme in the active oxygen scavenger system that dismutates $O_2^{\bullet -}$ into H_2O_2 which is further utilized by CAT and POD. Diamine oxidase (DAO) also produces H_2O_2 by catalysing oxidative deamination of the putrescine (Put) into H_2O_2 and pyrroline whereas polyamine oxidase (PAO) cleaves amino-propyl side chains at secondary amino group of spermidine (Spd) and spermine (Spm). In addition, their role in eliminating cellular polyamines (PAs) and the products formed from their action have been established (Su and Bai 2008). PAs have been differentially expressed in disease resistant and susceptible cultivars of tobacco (Marini *et al.* 2001). In developing barley grains, involvement of DAO in H_2O_2 production is required for POD-mediated lignin formation in chalazal cells (Asthir *et al.* 2002). Induced cellular lignification restricts pathogen entry into the cells and thus constitutes a typical defence reaction against a number of fungi, including rust fungi (Moldenhauer *et al*. 2006).

The hypersensitive response (HR) which is typical of resistant reaction serves as a trigger for the above events. This is followed by a cascade of signal transduction steps culminating in the expression of PR proteins. A relatively weaker induction of the cascade is observed in the susceptible host genotypes which lack the strong trigger provided by HR. In this study, the role of antioxidants and polyamines was investigated in a susceptible wheat cultivar at various levels of infection with stripe rust.

Resistant (PBW 343) and susceptible (HD 2329) cultivars of wheat (*Triticum aestivum* L.) were grown in pots filled with clay loam soil in the regular crop season following standard practices. Plants were inoculated at

Received 6 June 2008, *accepted* 25 March 2009.

Abbreviations: CAT - catalase; DAO - diamine oxidase; HR - hypersensitive response; PAs - polyamines; PAO - polyamine oxidase; POD - peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase.

* Corresponding author; fax (+91) 161 2400945, e-mail: b.asthir@rediffmail.com

seedling stage. A mixture of *P. striiformis* pathotypes prevalent in the region were used as fresh ureidial suspension (300 uredia cm^{-3}). Inoculated plants were then incubated at 13 ± 2 °C in opaque polythene chamber for 48 h to ensure saturated atmosphere for infection. Flag leaves of control plants as well as inoculated plants showing various infection stages ranging from traces to 100 % (0 to 100 S) were selected for laboratory studies.

Leaves (1 g) were homogenized in 5 cm^3 of ice-cold 50 mM phosphate buffer pH 6.5 (for POD, SOD), pH 7.5 (CAT) and pH 7.0 (DAO, PAO). The extracts were centrifuged at 10 000 *g* for 20 min at 0 to 4 °C in a *Beckmann* refrigerated centrifuge, and the supernatants were used for the enzyme activity assays. POD was assayed by the method of Claiborne and Fridovich (1979), CAT was measured using the method of Chance and Maehly (1955), whereas SOD was assayed by the method of Marklund and Marklund (1974). One unit (U) of SOD is the amount of enzyme that inhibits pyrogallol autooxidation by 50 %, monitored at 420 nm on *Elico* spectrophotometer (*UV-SL 159*). DAO and PAO were assayed following the method as described by Asthir *et al.* (2004). Ascorbate content was extracted and estimated as described by Gossett *et al*. (1994), tocopherol according to Hira *et al*. (2000) and polyamines according to Szczotka (1984). Chlorophyll content was extracted and estimated by the method of Hendry and Price (1993). Data from three replicates were statistically analyzed by a one-way analysis of variance. The differences were considered significant when $P \leq 0.05$.

Sporulation in the wheat-stripe rust system began at 6 to 7 d post inoculation and 'yellow islands' forming around infection sites were visible to the naked eye on the leaves of the susceptible cv. HD 2329 from this time onwards. In the resistant cv. PBW 343 there was no such visible formation of yellow islands at any stage following inoculation.

POD, SOD and CAT activities were higher in the leaves of resistant cv. PBW 343 than in the susceptible cv. HD 2329 in the controls (un-inoculated) (Table 1). However, after inoculation, all the three antioxidative enzymes increased in susceptible cv. showing their

Table 1. Effect of stripe rust on activities of POD [ΔA_{460} g^{-1}(f.m.) min^{-1}], SOD [U g^{-1}(f.m.)], CAT [ΔA_{240} g^{-1}(f.m.) min^{-1}], DAO [nmol(Δ-pyrroline) g^{-1}(f.m.) min^{-1}] and PAO [nmol(Δ-pyrroline) g^{-1}(f.m.) min^{-1}] in control leaves of resistant (PBW 343) and control and inoculated leaves of susceptible (HD 2329) wheat cultivars. Values are mean ± SD of three determinations. S - stands for susceptibility; TS, 10S, 20S, 30S, 40S, 60S, 80S, 100S stages are according to infected leaf area from traces (TS) to 100 % infection (100S).

Cultivars	POD	SOD	CAT	DAO	PAO
PBW 343	239 ± 9	20.3 ± 2.0	28.4 ± 1.9	72.1 ± 3.8	63.1 ± 2.5
HD 2329 control	198 ± 5	12.5 ± 1.3	14.3 ± 1.0	64.3 ± 2.7	52.3 ± 2.8
TS	229 ± 8	14.5 ± 1.3	17.2 ± 1.2	69.6 ± 2.5	56.5 ± 2.1
5S	258 ± 10	16.3 ± 1.2	19.5 ± 1.3	75.1 ± 3.1	63.1 ± 2.2
10S	295 ± 12	20.5 ± 1.5	22.3 ± 1.7	83.4 ± 3.3	72.4 ± 3.2
20S	345 ± 14	25.1 ± 1.4	26.1 ± 2.1	91.2 ± 3.7	80.1 ± 3.5
40S	393 ± 16	29.4 ± 2.1	31.4 ± 2.6	99.4 ± 4.3	86.4 ± 2.6
60S	320 ± 11	32.1 ± 2.7	40.1 ± 3.2	106.3 ± 4.5	91.3 ± 2.5
80S	208 ± 7	19.6 ± 1.4	21.3 ± 1.8	112.5 ± 5.1	97.1 ± 3.6
100S	168 ± 5	12.3 ± 1.1	9.1 ± 0.2	120.7 ± 5.3	111.0 ± 4.7

Table 2. Effect of stripe rust on contents of total ascorbate, α-tocopherol [μmolg^{-1}(f.m.)], chlorophyll (mg g^{-1}(f.m.)], polyamines (nmol g^{-1}(f.m.)] in leaves of resistant (PBW 343) and susceptible (HD 2329) wheat cultivars. For other detail see Table 1.

Cultivars	Ascorbate	α-Tocopherol	Chlorophyll	Putrescine	Spermidine	Spermine
PBW 343	97.4 ± 5.8	3.25 ± 0.080	124.6 ± 5.7	60.4 ± 3.0	32.3 ± 2.8	19.4 ± 1.5
HD 2329 control	78.6 ± 4.2	2.71 ± 0.072	114.5 ± 5.0	52.6 ± 2.4	26.1 ± 2.1	14.3 ± 1.2
TS	75.1 ± 4.8	2.62 ± 0.064	108.7 ± 5.9	63.1 ± 3.1	29.2 ± 2.2	17.2 ± 1.3
5S	73.3 ± 3.2	2.51 ± 0.058	99.3 ± 5.6	67.3 ± 3.2	37.4 ± 1.8	28.0 ± 1.6
10S	65.7 ± 2.8	2.38 ± 0.051	88.0 ± 5.2	72.7 ± 3.8	41.3 ± 2.4	35.7 ± 2.0
20S	59.2 ± 2.3	2.19 ± 0.042	74.3 ± 4.8	75.2 ± 2.3	47.1 ± 2.5	39.8 ± 1.2
40S	52.5 ± 2.1	2.04 ± 0.035	59.4 ± 3.7	81.4 ± 3.4	51.5 ± 2.3	45.6 ± 1.4
60S	43.6 ± 1.8	1.83 ± 0.027	44.5 ± 3.3	87.2 ± 2.2	58.2 ± 3.1	49.5 ± 1.6
80S	33.1 ± 1.4	1.60 ± 0.021	32.7 ± 3.2	95.3 ± 2.5	65.4 ± 3.4	57.4 ± 2.0
100S	25.4 ± 1.2	1.32 ± 0.012	24.7 ± 2.1	105.3 ± 2.7	69.1 ± 2.4	64.3 ± 2.2

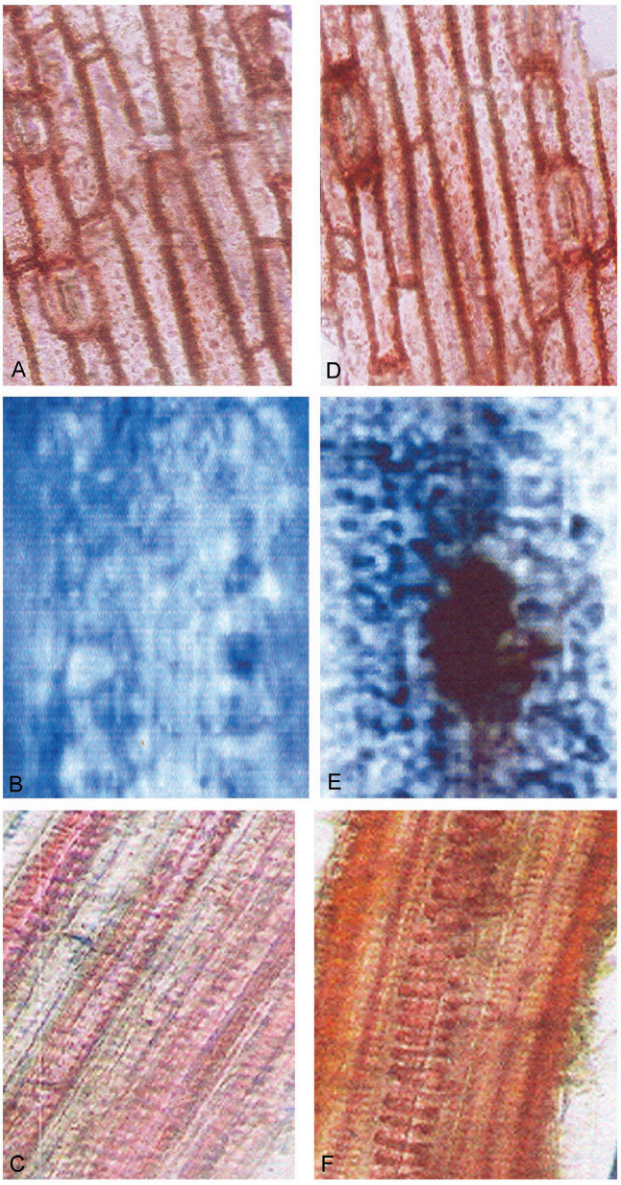

Fig. 1. Histochemical localization of POD (*A*, *D*), SOD (*B*, *E*) activities and lignin (*C*, *F*) content in inoculated leaves of PBW 343 (*A*, *B*, *C*) and HD 2329 (*D*, *E*, *F*).

maximum value at 40S - 60S stage. An increase in POD activity has been reported as a early response to plant pathogen interactions (Sasaki *et al.* 2004) and increased production of H_2O_2 was detoxified by POD and CAT. Similar results were reported in barley following powdery mildew (Harrach *et al.* 2005) and *Botrytis cinerea* (Kuzniak and Sklodowska 2005) infection. However, high SOD and CAT activities could not maintain at high infection of 80S - 100S, probably due to severe damage to cell machinery.

DAO and PAO activities showed consistent increase following infection and were highest at 100 S stage (Table 1). Activities of these enzymes were greater in the infected leaves than in the controls indicating their specific metabolic role in response to infection. The enhanced activities of these enzymes have been associated with resistance against, *e.g.*, powdery mildew in barley (Cowley and Walters 2002, Asthir *et al.* 2004) and *Ascochyta rabei* in chickpea (Angelini *et al.* 1993).

When leaf strips were incubated in acetate buffer containing 3 amino-9-ethyl carbazole, a dark reddish colour appeared near the cell walls of HD 2329, which showed POD activity (Fig.1*A,D*). As POD helps in lignin formation, so higher POD activity probably leads to higher lignin formation in infected leaves. Intense blue coloration was found in sections of HD 2329 showing higher SOD activity (Fig.1*B,E*). In cereals, induced cellular lignification has been shown to be an important mechanism of disease resistance against a number of fungi, including rust fungi (Moerschbacher and Mendgen 2000). Lignification has been implicated in rendering papillae more resistant to fungal penetration. In infected

wheat leaves, the appearance of pink colour may be a positive outcome of the phloroglucinol-HCl stain indicating the deposition of lignin or lignin like material in the necrotic cells. This structural defence reaction was more pronounced in resistant cv. PBW 343 than in susceptible cv. HD 2329.

The consistent increase in Put and Spd contents from 0 to 100S stages in HD 2329 (Table 2) suggests that these polyamines have a role in the resistance mechanism. Since H_2O_2 is a product of SOD, DAO and PAO activities, it seems likely that these enzymes may be responsible for the production of the H_2O_2 as a result of pathogen attack.

Put is a precursor of hydroxycinnamic acid and different alkaloids, which are known to be involved in resistance to pathogens (Liu *et al*. 2007). In addition, aminoaldehydes, which are derived from polyamine oxidation, may also be involved in the resistance mechanism as they are toxic to a variety of organisms. Supporting evidence for increased activity of polyamine synthesizing enzymes in wheat leaves after inoculation with *P. striiformis* comes from the work of Walters (2003), in which they presented evidence for increased activities of arginine decarboxylase, ornithine decarboxylase, and S-adenosylmethionine decarboxylase. Put, Spd, Spm contents were higher in control of PBW 343 as compared to HD 2329. Put was present in higher amount as compared to Spd and Spm in healthy and diseased leaves. Similar increase in PAs were also reported by Asthir *et al.* (2004) and extensively reviewed by Liu *et al.* (2007) indicating their ability to participate in stress tolerance. During the hypersensitive reaction (HR) against tobacco mosaic virus, polyamines were reported to increase around or in necrotic lesions (Yamakawa *et al.* 1998) in tobacco leaves.

Antioxidants like ascorbate, α-tocopherol along with chlorophyll content were higher in the un-inoculated leaves of PBW 343 than HD 2329 (Table 2). It has been reported earlier that ascorbic acid plays an important role in antioxidative reactions against necrotic processes associated with powdery mildew infection (EI-Zahaby *et al.* 1995). Low contents of ascorbate in response to pathogens were recorded by Barth *et al.* (2005). A decrease in α-tocopherol content following inoculation in HD 2329 suggests membrane instability. Ascorbate maintains the regeneration of α-tocopherol, providing synergetic protection of the membranes under stress conditions (Kanwischer *et al*. 2005). With the spread of disease, yellowing of leaves occurred which clearly showed decrease in chlorophyll content from 100 to 23 % from first to final stage (TS to 100 S) of infection in HD 2329 (Table 2). Similar decrease in chlorophyll under pathogen attack was reported by Kariola *et al.* (2005).

The study shows that in susceptible genotype there is a distinct biochemical response to disease. As a part of this response, POD, SOD, CAT, DAO and PAO activities increased as disease progressed, but physical damage to cell membranes led to rapid decline in content of ascorbate and tocopherol. An intriguing issue is whether pathogen metabolic system also contributed to the observed levels of antioxidants. This is an area of future research.

References

Agarwal, S., Sairam, R.K., Srivastava, G.C., Meena, R.C.: Changes in antioxidant enzymes activity and oxidative stress by abscisic acid and salicylic acid in wheat genotypes. - Biol. Plant. **49**: 541-550, 2005.

Anand, T., Bhaskaran, R., Raguchander, T., Samiyappan, R., Prakasam, V., Gopalakrishnan, C.: Defence responses of chilli fruits to *Colletotrichum capsici* and *Alternaria alternata*. - Biol. Plant. **53**: 553-559, 2009.

Angelini, R., Bragaloni, M., Federico, R., Infantino, A., Porta-Puglia, A.: Involvement of polyamines, diamine oxidase and peroxidase in resistance of chickpea to *Ascochyta rabiei.* - J. Plant Physiol. **142**: 704-709, 1993.

Asthir, B., Duffus, C.M., Smith, R., Spoor, W.: Diamine oxidase is involved in H_2O_2 production in the chalazal cells during barley grain filling. - J. exp. Bot. **53**: 677-682, 2002.

Asthir, B., Spoor, W., Duffus, C.M.: Involvement of polyamines, diamine oxidase and polyamine oxidase in resistance of barley to *Blumeria graminis* f. sp. *hordei* - Euphytica **136**: 307-312, 2004.

Barth, C., Moeder, W., Klessig, D.F., Conklin, P.L.: The timing of senescence and response to pathogens is altered in the ascorbate-deficient mutant vitamin C-1. - Plant Physiol. **134**: 1784-1792, 2005.

Chance, B., Maehly, A.C.: Assay of catalases and peroxidases. - Methods Enzymol. **2**: 764-775, 1955.

Claiborne, A., Fridovich, I.: Purification of the *o*-dianisidine peroxidase from *E. coli.* - J. biol. Chem. **254**: 4245-4252, 1979.

Cowley, T., Walters, D.R.: Polyamine metabolism in barley reacting hypersensitively to the powdery mildew fungus *Blumeria graminis* f. sp. *hordei*. - Plant Cell Environ. **25**: 461-468, 2002.

El-Zahaby, H.M., Gullner, G., Kiraly, Z.: Effects of powdery mildew infection of barley on the ascorbate-glutathione cycle and other antioxidants in different host-pathogen interactions. - Biochem. cell. Biol. **85**: 1225-1230, 1995.

Gossett, D.R., Millhollon, E.P., Lucas, M.C: Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. - Crop Sci. **34**: 706-714, 1994.

Harrach, B.D., Fodor, J., Barna, B.: Changes of antioxidants following powdery mildew infection of near-isogenic barley lines carrying different resistance genes. - Acta biol. Szeged. **49**: 91-92, 2005.

Hendry, G.A.F., Price, A.H.: Stress indicators: chlorophylls and carotenoids. - In: Hendry, G.A.F., Grime, J.P. (ed.): Methods in Comparative Plant Ecology: A Laboratory Manual. Pp. 148-152. Chapman and Hall, London 1993.

Hira, C.K., Kawatra, B.L., Sadana, B., Mann, S.K.: Laboratory Manual in Foods and Nutrition. - Punjab Agricultural University, Ludhiana 2000.

Kanwischer, I., Porfirova, S., Bergmuller, E., Dormann, P.: Alterations in tocopherol cyclase activity in transgenic and

mutant plants of *Arabidopsis* affect tocopherol content, tocopherol composition, and oxidative stress. - Plant Physiol. **137**: 713-723, 2005.

Kariola, T., Brader, G., Li, J., Palva, T.: Chlorophyllase I, a damage control enzyme, affects the balance between defense pathways in plants. - Plant Cell **17**: 282-294, 2005.

Kuzniak, E., Sklodowska, M.: Fungal pathogen-induced changes in the antioxidant systems of leaf peroxisomes from infected tomato plants. - Planta **22**: 192-200, 2005.

Liu, J.H., Kitashiba, H., Wang, J., Ban, Y., Moriguchi T.: Polyamines and their ability to provide environmental stress tolerance to plants. - Plant Biotechnol. **24**: 117-126, 2007.

Liu, Q., Yang, J.L., He, L.S., Li, Y.Y., Zheng, S.J.: Effect of aluminium on cell wall, plasma membrane, antioxidants and root elongation in triticale. - Biol. Plant. **52**: 87-92, 2008.

Marini, F., Betti, L., Scaramagli, S., Biondi, S., Torrigiani, P.: Polyamine metabolism is upregulated in response to tobacco mosaic virus in hypersensitive, but not in susceptible tobacco. - New Phytol. **149**: 301-309, 2001.

Marklund, S., Marklund, G.: Involvement of superoxide anion radical in the auto-oxidation of pyrogallol and a convenient assay for superoxide dismutase. - Eur. J. Biochem. **47**: 169-174, 1974.

Moerschbacher, B.M., Mendgen, K.: Structural aspects of defense. - In: Slusarenko, A., Fraser, R.S.S., Van Loon, L.C. (ed.): Mechanisms of Resistance to Plant Diseases. Pp. 231-237. Kluwer Academic Publishers, Dordrecht 2000.

Moldenhauer, J., Moerschbacher, B.M., Van der Westhuizen, A.J.: Histological investigation of stripe rust (*Puccinia striiformis* f. sp. *tritici*) development in resistant and susceptible wheat cultivars. - Plant Pathol. **55**: 469-474, 2006.

Sache, I., De Vallavieille-Pope, C.: Comparison of the wheat brown and yellow rusts, for monocyclic sporulation and infection processes, and their polycyclic consequences. - J. Plant Pathol. **138**: 55-65, 1993.

Sasaki, K., Iwai, T., Miraga, S., Kuroda, K., Seo, S., Mitsuhara, I., Miyasaka, A., Iwamo, M., Ito, H., Matsui, H.: Ten rice peroxidases redundantly respond to multiple stresses including infection with rice plant fungus. - Plant Cell Physiol. **45**: 144-152, 2004.

Su, G.X., Bai, X.: Contribution of putrescine degradation to proline accumulation in soybean leaves under salinity. - Biol. Plant. **52**: 796-799, 2008.

Suzuki, N., Mittler, R.: Reactive oxygen species and temperature stresses: A delicate balance between signalling and destruction. - Physiol. Plant. **126**: 45-51, 2006.

Szczotka, Z.: Polyamine changes in (*Quercus borealis* Michx.) and (*Quercus robur* L.) seeds during ageing in controlled conditions. - Acta Physiol. Plant. **6**: 127-135, 1984.

Walters D.: Resistance to plant pathogens: possible roles for free polyamines and polyamine catabolism. - New Phytol. **159**: 109-115, 2003.

Yamakawa, H., Kamada, H., Satoh, M., Ohashi, Y.: Spermine is a salicylate-independent endogenous inducer in both tobacco acidic pathogensis-related proteins and resistance against tobacco mosaic virus infection. - Plant Physiol. **118**: 1213-1222, 1998.