ANTIOXIDANT ENZYMES AND ALDEHYDE RELEASING CAPACITY OF RICE CULTIVARS (*ORYZA SATIVA* L.) AS DETERMINANTS OF ANAEROBIC SEEDLING ESTABLISHMENT CAPACITY

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Summary. The activities of antioxidant enzymes and levels of antioxidants involved in oxygen detoxification were studied in seedlings of three indica rice (*Oryza sativa* L.) cultivars, namely Panikekoa and T 1471 that show good capacity for anaerobic seedling establishment (tolerant reaction) and IR 42 which has a poor anaerobic seedling establishment capacity (susceptible reaction). The activities of the antioxidant enzymes tested decreased under submergence. The activity of glutathione reductase (GR, EC 1.6.4.2) was found to be more sensitive compared to the other enzymes. On the other hand, the activity of superoxide dismutase (SOD, EC 1.15.1.9) was comparatively stable. After 5 days of submergence tolerant cultivars maintained almost 3-fold higher glutathione reductase activity than the susceptible cultivar. During submergence the tolerant cultivars showed comparatively less ascorbic acid oxidase (AAO, EC 1.10.3.3) activity and had greater ascorbic acid content. The activities of superoxide dismutase, catalase (CAT, EC 1.11.1.6), peroxidase (POX, EC 1.11.1.7), ascorbic acid peroxidase (APX, EC 1.11.1.11) and polyphenol oxidase (PPO, EC 1.14.18.1) were also higher in tolerant cultivars during submergence compared to the susceptible cultivar. Submergence was found to induce aldehyde releasing capacity in rice seedlings which was more apparent in the susceptible cultivar.

Keywords*:* aldehyde, anaerobic seedling establishment, antioxidant enzyme, rice

Abbreviations**:** SOD- superoxide dismutase, CAT- catalase, POX- peroxidase, APX- ascorbic acid peroxidase, GR- glutathione reductase, PPO- polyphenol oxidase, AAO- ascorbic acid oxidase, AA- ascorbic acid, MBTH- 3-methyl-2-benzothiazolinone-hydrazine, DW- dry weight

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INTRODUCTION

Direct seeding of rice is common in rainfed lowlands as apart from being economic it induces robustness in seedlings enabling them withstand complete submergence during heavy rains. However, seedling establishment is extremely poor if seeding is followed soon by substantial rains. An alternative is to raise seedling in a nursery and transplant them at a suitable age although complete wash out due to heavy rains may still not be ruled out. Some rice cultivars grown in rainfed lowlands of eastern India have been found to tolerate flooding during early seedling establishment (Sarkar et al., 1999; Sarkar and Das, 2003), presumably because of their faster growth rate and ability to survive under low light intensity conditions to which the seedlings submerged under turbid water for an initial period of 3 to 4 days. The mechanism that endows these cultivars with the capacity to establish them at the seedling stage and survive under submergence remains unknown. Though, rice is relatively tolerant to anaerobiosis compared to other cereals like maize and wheat (Mustroph and Albrecht, 2003), some of the rice cultivars are known to withstand and survive better under anaerobic conditions caused by submergence.

The sudden burst of oxygen to which the plants are exposed as the floodwater recede has a detrimental effect on them. Many rhizomatous species from wetland habitat are naturally well-protected against such post-anoxic injury. Some enzymes are known to be induced under hypoxic/anoxic conditions that have a protective role to play upon subsequent exposure of plants to oxygen (Monk et al., 1987a). Anoxia-tolerant species like lotus (*Nelumbo nucifera*) are known to germinate, grow and maintain similar levels of activities of certain anti-oxidant enzymes under hypoxia as compared to the plants grown under normal conditions (Ushimaru et al., 2001). On the other hand the activities of antioxidant enzymes in submergence sensitive *Japonica* cultivars of rice decrease substantially under submergence (Ushimaru et al., 1999). Information is lacking on the changes in anti-oxidant enzymes activities and antioxidant levels in more widely grown *Indica* rice cultivars some of which have been found to establish themselves in seedling stage even under hypoxic conditions, created by submergence.

Energy needs of plants under submergence are met through alcoholic fermentation. Though levels of ethanol so produced rarely exceed toxic levels in submerged rice seedlings (Ellis and Setter, 1999): levels of acetaldehyde, a known toxicant and precursor of ethanol increase sharply during the post anoxic period when the flood water recedes (Rahman et al., 2001). This has been attributed to the NAD^+-dependent conversion of ethanol to acetaldehyde catalyzed by alcohol dehydrogenase and to H_2O_2-dependent catalase –controlled peroxidation (Monk et al., 1987a; 1987b). Variability in cultivars with respect to their capacity to produce acetaldehyde, a toxic substance may be a factor that determines their anaerobic seedling establishment capacity.

The objective of the present study, therefore, was to get an insight of how levels of some natural anti-oxidants and the activities of important anti-oxidant enzymes vary during hypoxic conditions in *Indica* rice cultivars differing in their capacity for anaerobic seedling establishment. In addition, aldehyde releasing capacity of submerged seedlings was also studied to find if the trait affected anaerobic seedling establishment in any way.

MATERIAL AND METHODS

The study was conducted using three contrasting *indica* rice (*Oryza sativa* L) cultivars namely, IR 42 which is poor at anaerobic seedling establishment and T 1471 and Panikekoa, both endowed with better anaerobic seedling establishment capacity. Seeds of each cultivar were surface sterilized with 0.1% $HgCl_2$ solution for 5 minutes and thoroughly washed with sterilized distilled water and later allowed to germinate in petri dish at 30ºC. The 72 h old seedlings so obtained were completely submerged for 5 days with water level 20 cm above the seedlings in glass beakers and incubated at 25-30ºC for initial 3 days in darkness (to simulate low light conditions of flood water) followed by exposure to diffuse light for remaining two days. Non-submerged seedlings grown under normal conditions formed the control sets. After this period the water level was brought down such that only the roots remain submerged. The seedlings were now allowed to grow for another seven days under laboratory conditions. The plants that did not decompose and remained green were considered as survivors. Survival in tolerant cultivars (e.g. Panikekoa and T 1471) was above 85 percent whereas susceptible cultivar (e.g. IR 42) was totally decomposed (data not given). The experiment was repeated three times. The average data of the three replications were used for statistical analysis using a randomized complete block design (RCBD) model following IRRISTAT package.

The dissolved oxygen levels were determined at 12 noon at 10 cm water depth after 1, 3, and 5 days of submergence with a Simplair-F-5, Syland Scientific GMBH, Heppenheim 1, Germany.

A 500 mg shoot sample was homogenized in 10 ml of 0.1 M potassium phosphate buffer, pH 7.8, containing 1 % insoluble polyvinylpyrrolidone. The extract was centrifuged at 4ºC at 15000 g for 30 min, and the supernatant was used for assaying the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and polyphenol oxidase (PPO). The SOD activity was assayed according to Giannopolitis and Ries (1977) with modification suggested by Chowdhury and Chowdhuri (1985). The activities of CAT and POX (Ram et al., 2000), PPO (Sarkar et al., 2001), GR (Foster and Hess, 1980), APX (Nakano and Asada, 1981) and AAO (Olliver, 1987) were estimated following the normal procedures described earlier.

Ascorbic acid (AA) was extracted from the shoot tissue in 4% oxalic acid at 4ºC.

Supernatant measuring 5 ml was taken in a 50ml volumetric flask and 10ml of oxalic acid was added to it (Sadasivam and Balasubraminan, 1987). The flask was swirled and titrated against 2-6-DCPIP solution (42 mg sodium bicarbonate + 0.52 mg 2-6-DCPIP made up to 200ml with distilled water and diluted 1:1 with water) taken in a burette. The end point of titration was the appearance of pink colour that persisted for at least 30 s. The ascorbic acid content was determined from the standard curve made with known concentration of L-ascorbic acid.

The aldehyde content of seedlings was measured following the method of Kundu et al. (1993) with some modification (Sarkar, 2001). Rice seedlings were placed in an air tight 30 ml test tube containing 10 ml of 0.2% (w/v) 3-methyl-2-benzothiazolinone-hydrazine (MBTH). The tubes with the seedlings were incubated in dark at 25-30^{o}C for 48 h. The aldehyde trapped by the MBTH was determined by drawing 0.2 ml aliquot from each tube that was mixed with 2.6 ml 0.2% MBTH and 2.5 ml 0.23% $FeCl_3$ (w/v) in fresh test tubes and incubated at 30^{o}C for 10 min followed by addition of 2.5 ml acetone and subsequent closure of test tubes with tightly fit corks. Absorbance at 635 nm was measured after 30 min. Formaldehyde solution was used to prepare a standard curve.

Results and discussion

Oxygen concentration decreased with increased period of submergence (Fig. 1), the magnitude being most pronounced during the first 24 h of submergence. This was apparently because of the continuous utilization of dissolved oxygen by seedlings

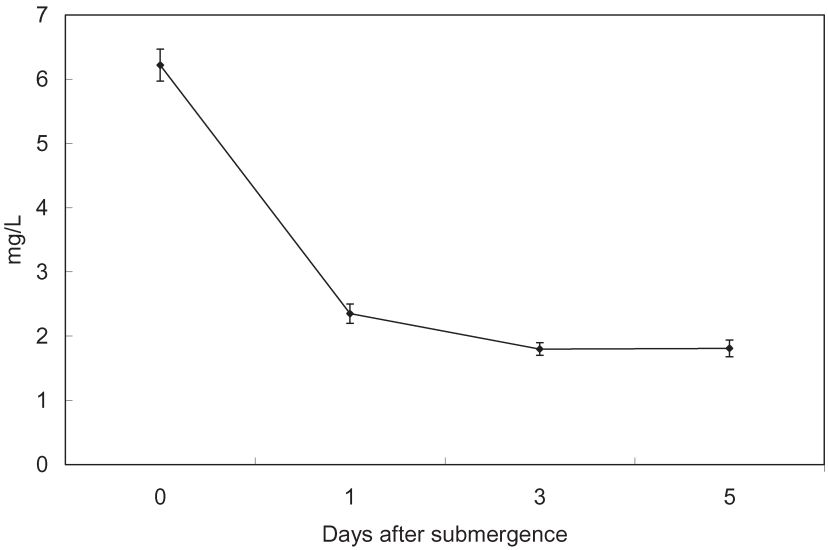

Fig. 1. Variation in oxygen concentration in flood water with duration. Bar represents means ± standard deviation.

and the lack of oxygenic photosynthesis. As diffusion of gases in water is 10,000 times less compared to that in air (Armstrong, 1979), diffusion from air was unlikely to increase the level of oxygen in water. Hence, plants experienced a perfect hypoxic condition 2 mg L^{-1} (air equilibrium 7.24 mg L^{-1} at 30°C).

Under control condition the three cultivars more or less maintained similar levels of enzyme activities. Submergence caused reduction in activities of antioxidant enzymes in both tolerant and susceptible cultivars, which was more apparent after 1 day of submergence (Table 1). Significant differences of the activities of antioxidant enzymes were noticed among the varieties and within the periods of submergence. On the other hand, under submergence the activities of all the antioxidant enzymes namely SOD, CAT, POX, PPO, GR and APX were significantly greater in tolerant cultivars compared to the susceptible cultivar. Variety x treatment interaction was also significant. Study of normalized values (value of submerged sample/value of non-submerged control sample) revealed that among the antioxidant enzymes, the activity of SOD was less affected under submergence whereas the activity of GR was most sensitive to submergence. Maintenance of higher activity of SOD both in submergence tolerant and susceptible rice cultivars has been reported earlier (Sarkar and Das, 2000; Sarkar et al., 2001). The reduction in antioxidant enzyme activities in control (non-submerged) seedlings was possibly due to the incubation of seedlings in darkness which restricted oxygenic photosynthetic reaction while under submergence the higher reduction in the activities of the antioxidant enzymes was due to the lack of light and hypoxic environment (Fig. 1).

The activities of CAT, POX and PPO were significantly higher in tolerant cultivars than the susceptible cultivar (Table 1B, 1C, 1D). Rice seedlings that grow under water or those which get submerged after normal growth can utilize molecular oxygen in water (Ushimaru et al., 1992a; Sarkar and Bera, 1997). Reduction in the activities of GR and APX has been reported in wheat (Biemelt et al., 1998) and rice (Ushimaru et al., 1997) seedlings during anoxia. The increased activities of these enzymes in tolerant cultivars emphasize better physiological adaptation of these cultivars under hypoxic condition, so as to maintain the structural integrity of enzyme

Table 1. Changes of some anti-oxidant enzymes due to submergence

A. Superoxide dismutase (unit/g DW)

Days after	Panikekoa			T 1471			IR 42		
submergence	C	S	N	C	S	N	C	S	N
1	205a	114c	0.56	224a	92c	0.41	208a	91c	0.44
3	182b	126b	0.69	193b	133b	0.69	189b	109b	0.58
5	173c	142a	0.82	172c	148a	0.86	162c	123a	0.76
Variety x Treatment	*P< 0.05 = 4.8, *P< 0.01 = 6.6								

B. Catalase (unit/min/g DW)

Days after	Panikekoa			T 1471			IR 42		
submergence	C	S	N	C	S	N	C	S	N
1	51.05a	15.45c	0.30	47.45a	15.30c	0.32	53.45a	11.35b	0.21
3	37.85b	18.40b	0.49	36.20b	18.35b	0.51	38.35b	12.95ab	0.34
5	30.95c	22.75a	0.73	30.30c	21.20a	0.70	31.10c	14.45a	0.46
Variety x Treatment	*P< 0.05 = 2.40, *P< 0.01 = 3.29								

C. Peroxidase (unit/min/g DW)

Days after	Panikekoa			T 1471			IR 42		
submergence	C	S	N	C	S	N	C	S	N
1	17.55a	4.15c	0.24	17.80a	4.45c	0.25	18.45a	2.65c	0.14
3	14.50b	6.65b	0.46	15.05b	6.50b	0.43	14.90b	4.60b	0.31
5	12.45c	9.50a	0.54	12.55c	9.05a	0.72	13.35c	5.60a	0.42
Variety x Treatment	*P< 0.05 = 0.72, *P< 0.01 = 0.99								

D. Polyphenol oxidase (unit/min/g DW)

Days after	Panikekoa			T 1471			IR 42		
submergence	C	S	N	C	S	N	C	S	N
1	83.60a	28.55c	0.34	80.40a	28.15c	0.35	83.70a	19.55c	0.23
3	79.55b	34.55b	0.44	75.95b	33.60b	0.43	75.50b	27.50b	0.36
5	69.80c	44.10a	0.63	68.85c	43.05a	0.64	69.20c	30.65a	0.44
Variety x Treatment	*P< 0.05 = 3.23, *P< 0.05 = 4.44								

E. Glutathione reductase (mmol/min/g DW)

Days after	Panikekoa			T 1471			IR 42		
submergence	C	S	N	C	S	N	C	S	N
1	18.72a	7.18a	0.38	18.15a	8.23a	0.45	17.12a	5.21a	0.30
3	16.64a	8.20a	0.49	16.17a	8.01a	0.49	16.19a	3.23ab	0.20
5	16.08a	6.53a	0.41	15.42a	5.93a	0.38	15.94a	2.07b	0.13
Variety x Treatment	*P< 0.05 = 2.75, *P< 0.01 = 3.78								

F. Ascorbic acid peroxidase (mmol/min/g DW)

Days after	Panikekoa			T 1471			IR 42		
submergence	C	S	N	C	S	N	C	S	N
1	19.25a	4.60c	0.24	19.25a	4.50c	0.23	18.90a	2.60c	0.14
3	16.55b	6.35b	0.38	16.40b	6.10b	0.37	16.45b	3.90b	0.24
5	14.75c	9.05a	0.61	13.80c	9.40a	0.68	14.55c	5.25a	0.36
Variety x Treatment	*P< 0.05 = 0.77, *P< 0.01 = 1.06								

In a column, means followed by a common letter are not significantly different at the 5% level by Duncan Multiple Range Test. C= control, S=submerged, N= normalized value (S/C).

molecules better and when needed upon exposure to high oxygen concentration detoxify the toxic oxygen species and protect the system effectively.

Under submergence, the activities of AAO were more in susceptible cultivar, which had lower concentration of ascorbic acid (AA) (Table 2). The enzyme AAO directly oxidizes ascorbic acid in presence of molecular oxygen resulting in dehy-

Table 2. Changes of ascorbic acid oxidase and ascorbic acid content due to submergence

A. Ascorbic acid oxidase (mol ascorbic acid decomposed /30 min /g DW)

Days after	Panikekoa			T 1471			IR 42		
submergence	C	S	N	C	S	N	C	S	N
1	128.60a	55.50c	0.43	124.90a	56.95c	0.45	126.50a	77.75b	0.61
3	115.45b	70.15b	0.61	115.95b	71.80b	0.62	117.75b	91.40a	0.78
5	102.80c	84.50a	0.82	104.40c	83.05a	0.79	106.45c	96.15a	0.90
Variety x Treatment	*P< 0.05 = 5.02, *P< 0.01 = 6.89								

B. Ascorbic acid content (mol ascorbic acid /g DW)

Days after	Panikekoa			T 1471			IR 42		
submergence	C	S	N	C	S	N	C	S	N
1	82.7b	139.5a	1.69	82.4b	126.6a	1.54	82.3b	109.4a	1.33
3	97.5a	117.5b	1.20	96.3a	118.4b	1.23	93.1a	87.3b	0.94
5	97.9a	124.8b	1.27	90.4ab	123.4ab	1.36	71.6c	71.6c	1.00
Variety x Treatment	*P< 0.05 = 5.92 , *P< 0.01 = 8.14								

In a column, mean values followed by a common letter are not significantly different at the 5% level by Duncan Multiple Range Test. C= control, S=submerged, N= normalized value (S/C).

droascorbic acid, which is converted to AA by dehydroascorbate reductase (DHAR). The activities of DHAR are low in rice seedlings that germinate under water (Ushimaru et al., 1992b) and thus might cause lowering of the AA content when submergence is prolonged. Besides, the maintenance of higher content of AA in tolerant cultivar might be attributed to the lower AAO activity here in comparison to the susceptible cultivar. Biemelt et al. (1998) reported higher levels of ascorbate under hypoxia in wheat root system, while upon re-aeration it declined. In the present case also AA accumulated under submergence; the extent of accumulation was greater in tolerant cultivars (Table 2B). Function of ascorbic acid oxidase is yet to be understand properly (Tullio et al., 2004), yet we can hypothesized from this investigation that with repression of AAO and the increase of AA content which is a powerful antioxidant might help in detoxifying the oxygen species and thus tolerant cultivars accumulated more amount of AA than susceptible cultivar.

Under submergence the aldehyde releasing capacity was more in susceptible cultivar compared to the tolerant cultivars (Fig. 2) indicating that aldehyde releasing capacity may form the basis of distinguishing tolerant cultivar from the susceptible one. Overproduction of aldehyde might be the cause of the death of the susceptible cultivar. Sarkar (2001) reported a highly significant negative association between submergence tolerance and aldehyde releasing capacity in twenty one-day-old rice seedlings in fourteen rice cultivars.

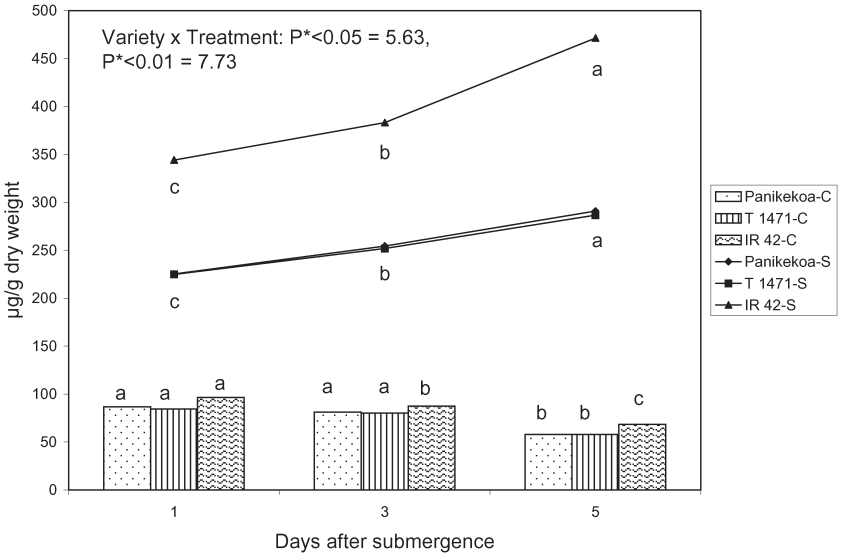

Fig. 2. Changes of aldehyde releasing capacity due to submergence. In a treatment for a particular cultivar followed by a common letter is not significantly different at 5% level by Duncans Multiple Range Test. There were no significant differences in tolerant cultivars either in controlled condition or in submerged condition. C= control, S= submerged. Column graphs represent non-submerged controlled samples whereas line graphs represent submerged samples.

It is important that antioxidant enzymes should work together, for providing better protection of plants during the time of oxidative stress. Ascorbate and glutathione remove H_2O_2 via the Halliwell-Asada pathway (May et al., 1998). Ascorbate serves not only to reduce H_2O_2 in Halliwell-Asada pathway but also helps in detoxifying the H_2O_2 in vacuoles through peroxidase/phenolics/ascorbate system (Yamasaki and Grace, 1998). Both systems are in operation in plants. The peroxidase/phenolics/ascorbate system is specific for plant tissues (Blokhina et al., 2003). The present investigation suggests that under submergence, tolerant cultivars adapt themselves in such a way that their antioxidant system is maintained at a higher level to detoxify the reactive oxygen species that form after hypoxia. The ascorbate related antioxidant system plays an important role in ensuing plant survival. The study also indicates that a direct correlation exists between submergence susceptibility (poorer capacity for anaerobic seedling establishment) and aldehyde releasing capacity of a cultivar.

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