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Changes in Certain Antioxidative Enzymes and Growth Parameters as a Result of Complete Submergence and Subsequent Re-aeration of Rice Cultivars Differing in Submergence Tolerance

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With 2 figures and 3 tables

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Abstract

An experiment was conducted on the three *indica* rice (*Oryza sativa* L.) cultivars IR 42, CR 383-10 and FR 13A, which are susceptible to submergence, submergence-avoiding and tolerant to submergence, respectively. A deleterious effect of submergence was noted as both above-ground dry matter accumulation and chlorophyll content decreased during submergence and subsequent re-aeration. However, the rate of reduction was lower in the tolerant cultivar. The tolerant cultivar FR 13A maintained higher dry weight during submergence and subsequent re-aeration and accumulated lower proline and malondialdehyde contents than the other cultivars. The activities of the enzymes catalase, peroxidase and superoxide dismutase, expressed on a per g fresh weight of leaves basis, were higher in FR 13A than in the other cultivars, both under water and 24 h after the initiation of re-aeration. However, ascorbic acid oxidase activity was lower in FR 13A. The cultivar CR 383-10, which has the capacity to accumulate more above-ground dry matter under normal conditions, showed a greater elongation ability under submergence and was similar to the susceptible cultivar IR 42 in terms of enzyme activities and the other parameters investigated during submergence and subsequent re-aeration.

Key words: antioxidative enzymes — Gramineae — *Oryza sativa* L. — submergence tolerance

Introduction

Complete submergence of lowland rice crops during flash floods occurs in large areas of South-East Asia, when plants may be completely under water for up to 1–2 weeks, resulting in an increased mortality of plants and low grain yield. A total of 22 million hectares of rice-growing area is adversely affected by flash flooding, half of which is in eastern India (Roy 1993). Submerged plants experience low oxygen and low light intensity under water relative to that in air. Because no plants are known to survive indefinitely under complete submergence, an ability to deal with the consequences of re-exposure to oxygen (O_2) and high light intensity is important when post-hypoxic injury causes plant mortality (Drew 1997, Ito et al. 1999). Under hypoxia, enzymes are induced that have a role in plant metabolism during subsequent exposure of plants to O_2 (Monk et al. 1987). The reports published to date deal with species mainly grown in wetland ecology. In the case of rice, O_2 detoxification during re-aeration may also be important in withstanding submergence stress. This paper reports changes in certain antioxidative enzymes and growth parameters under complete submergence and subsequent shifting of submerged seedlings to normal conditions in rice cultivars that differ in submergence tolerance.

Materials and Methods

Plant material and growth conditions

The experiment was conducted on three *indica* rice (*Oryza sativa* L.) cultivars: IR 42 (susceptible to submergence), CR 383-10 (submergence-avoiding) and FR 13A (tolerant to submergence) (Sarkar et al. 1998). All the cultivars were sown directly in earthenware pots containing 2 kg of farm soil and farmyard manure in a 3 : 1 ratio. Each pot was supplied with 80 mg urea, 192 mg single super phosphate (P_2O_5) and 70 mg murate of potash (K_2O). Ten days after germination, seedlings were thinned and three plants per pot were maintained.

Twenty-day-old seedlings were completely submerged in concrete tanks at a depth of 110 cm of water. Three different treatments were tried: (1) under water for 8 days (submerged); (2) under water for 8 days and then in air for 1 day (air adaptation) and (3) air-grown control plants. The experiment was repeated three times. Regeneration capacity was measured by counting the number of surviving plants 10 days after the beginning of re-aeration. The characteristics of the floodwater in terms of light transmission (%) were measured at 1200 h (LI-COR, Lincoln, NB), and water temperature and oxygen concentration were determined at 0600 and 1200 h (Syland, Heppenheim, Germany). Light intensity at 30 and 60 cm water depth varied between 60 and 64 % and 43 and 46 %, respectively, of the incident irradiance above the floodwater. The oxygen concentration at the same water depth was 1.8–2.1 p.p.m. at 0600 h and 4.8–5.3 p.p.m. at 1200 h. The temperature did not vary greatly, being 25.4–30.6 °C throughout the period of the experiment.

Extraction and assay of antioxidant enzymes

A 500-mg sample of leaves was homogenized in 5 ml of grinding medium prepared for each enzyme, as mentioned below. The extract was centrifuged at 4 °C at 15 000 ***g*** for 20 min, and the supernatant was used for assays. All operations were performed under a dim green light.

Superoxide dismutase (SOD)

For the determination of SOD activity, the enzyme was extracted in 0.1 M potassium phosphate buffer (pH 7.8) containing 1 % (w/v) insoluble PVPP. The enzyme activity was determined by measuring its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT) following Giannopolitis and Ries (1977) with modifications suggested by Chowdhury and Choudhuri (1985). The 3-ml reaction mixture contained 0.05 M Na_2CO_3, 0.1 mM EDTA, 63 μM NBT, 13 μM methionine, 0.2 ml enzyme extract and 1.3 μM riboflavin. The riboflavin was added last. The test-tubes were placed under two 40-W fluorescent lamps at a distance of 30 cm at 25 °C. After 15 min, the light was switched off and the absorbance at 560 nm was noted. The non-irradiated sample served as a control and was deducted from A_{560}. The reaction mixture without the enzyme developed maximum colour due to maximum photo-reduction of NBT. The reduction of NBT was inversely proportional to the enzyme activity. Thus, to obtain the activity, the A_{560} of a particular set was deducted from the A_{560} of the blank set (without enzyme).

Catalase (CAT)

The enzyme was extracted in the SOD grinding medium. The reaction mixture consisted of 5 ml phosphate buffer (0.05 M, pH 7.4), 1 ml H_2O_2 (46.9 mM) and 0.2 ml of enzyme. The reaction mixture was incubated for 5 min at 30 °C. Degradation of H_2O_2 was determined by the method described previously (Sagisaka 1976). The test-tubes contained 0.4 ml of 50 % trichloroacetic acid, 0.4 ml of 10 mM ferrous ammonium sulphate and 0.2 ml of 2.5 M potassium thiocyanate in which 1.6 ml of reaction mixture was added for colour development and was read at 480 nm. The difference between the readings at 0 and 3 min incubation determined the degradation of H_2O_2.

Peroxidase (PER)

The enzyme was extracted in SOD grinding medium. The reaction mixture consisted of 0.2 ml of enzyme, 5 ml phosphate buffer (0.05 M, pH 6.0), 1 ml H_2O_2 (46.9 mM) and 1 ml catechol (0.5 %). PER was assayed by the method of Chance and Maehly (1955), whereby colorimetric determination of the change in the colour intensity of oxidized catechol at 420 nm was recorded.

Ascorbic acid oxidase (AAO)

Ascorbic acid oxidase activity was assayed according to Olliver (1967). The enzyme was extracted in phosphate buffer (0.05 M, pH 7.4). The reaction mixture consisted of 1 ml crude extract, 2 ml ascorbic acid (2 mM) and 3 ml extracting buffer. It was incubated for 30 min at 37 °C. A blank set was prepared by deducting 1 ml enzyme from the reaction mixture and replacing it with 1 ml of phosphate buffer. The reaction was stopped with 5 ml of 10 % TCA and the reaction mixture was titrated with DCPIP (2,6-dichlorophenol indophenol). The difference between the two readings gave the ascorbic acid oxidase activity.

Estimation of chlorophyll

For chlorophyll estimation, 100 mg of finely chopped leaves was put in a 25-ml capped measuring

tube containing 80 % acetone, and kept inside a refrigerator (4 °C) for 28 h. The chlorophyll was measured spectrophotometrically following Arnon (1949).

Estimation of malondialdehyde (MDA)

A 500-mg sample of leaves was extracted with 1 % (w/v) trichloroacetic acid (TCA) and MDA content was determined by adding an equal aliquot of 0.5 % thiobarbituric acid in 20 % TCA to an aliquot of the extract according to Heath and Packer (1968). The solution was heated at 95 °C for 25 min. Absorbance was measured at 532 nm, corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The amount of MDA was calculated by using an extinction coefficient of 155^{-1} cm^{-1}.

Estimation of proline

A 500-mg sample of leaves was crushed in 3 % sulphosalicylic acid and proline was estimated following the methods of Bates et al. (1973).

Estimation of protein

Proteins were precipitated from stock crude extracts of enzymes with the same volume of 18 % (w/v) TCA at 4 °C. Then the precipitate was dissolved in 1N NaOH for protein measurement following Lowry et al. (1951). Bovine serum albumin was used as standard.

Results and Discussion

In general, submergence increased elongation ability and so plants had greater height after submergence. Total elongation after submergence was highest in CR 383-10, a submergence-avoiding genotype (Fig. 1). The elongation capacities of the submergence-tolerant and -susceptible cultivars were similar. However, there was a large difference in regeneration capacity, with the submergence-tolerant cultivar FR 13A showing 100 % survival.

Before submergence, the total above-ground dry matter per plant was highest in CR 383-10, followed by FR 13A, which were statistically non-significant (Fig. 2a). Under submergence, dry matter accumulation decreased, but after 3 days of re-aeration only the tolerant cultivar FR 13A showed an upward trend of dry matter accumulation. Like dry matter accumulation, the chlorophyll content of

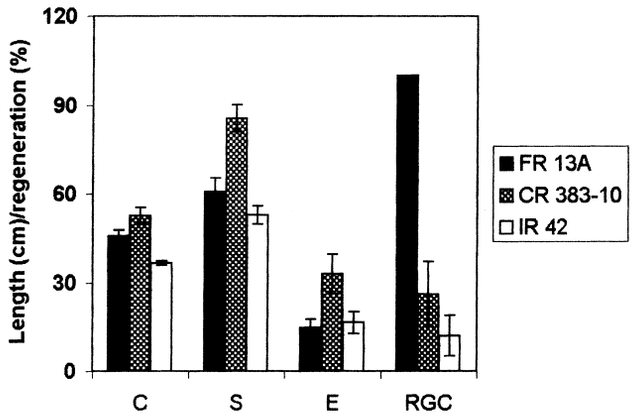

Fig. 1: Changes of plant height and regeneration capacity (RGC) due to submergence and subsequent re-aeration. Bars indicate standard deviation. C = control; S = submerged; E = elongation caused by submergence

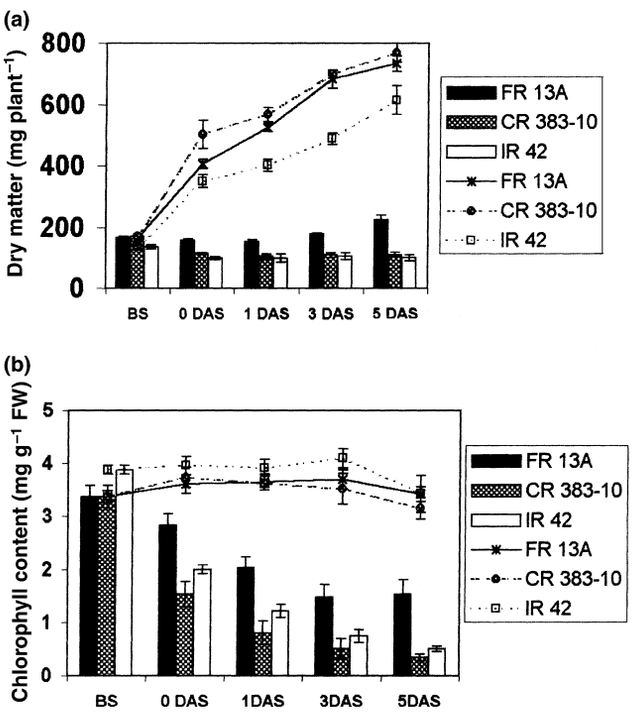

Fig. 2: Changes of dry matter and chlorophyll content with growth. BS = before submergence; DAS = days after start of re-aeration. The line graph represents control seedlings, and the histogram submerged seedlings; bars indicate standard deviation

the leaves decreased not only during submergence but also during re-aeration (Fig. 2b). The chlorophyll content was always highest in the tolerant cultivar FR 13A during submergence and in plants that experienced submergence earlier. Under control conditions, however, chlorophyll content was highest in the susceptible cultivar IR 42. Maintenance of more chlorophyll and dry matter content

during submergence and subsequent re-aeration might contribute to the higher regeneration capacity exhibited by FR 13A.

In general, submergence reduced the activities of all the antioxidative enzymes. However, after exposure to air, the activities of the enzymes increased (Tables 1 and 2). The activity of PER, however, did not increase during re-aeration in IR 42 and CR 383-10. The activities of CAT, PER and SOD were very similar in the tolerant cultivar FR 13A and the susceptible cultivar IR 42 under control conditions. However, during submergence and after exposure to air, the activities of these enzymes were higher only in the tolerant cultivar FR 13A in comparison to the other two cultivars. The activity of SOD increased on transfer from submerged to air-adapted conditions, although it remained lower than that of the aerobically grown control. Ushimaru et al. (1992) reported that antioxidative enzymes were co-regulated such that they showed higher activities at higher O_2 tensions. It appeared that after re-aeration the activities of O_2 detoxification enzymes in previously submerged seedlings were restored to some extent, especially in the tolerant cultivar FR 13A.

The activities of CAT and PER were higher under submerged conditions in the flooding-tolerant cultivar FR 13A than in CR 383-10 and IR 42. The greater activities of CAT and PER under submergence, especially in the tolerant cultivar, were possibly due to higher absorption of O_2 from the surrounding water. Sarkar and Bera (1997) reported that the tolerant cultivar had higher root activity, as measured by α-naphthylamine oxidation, and hence could transport more O_2 through the shoot to the roots under complete submergence.

Unlike other enzyme activities, the activity of AAO was lower in the tolerant cultivar FR 13A than in the other two cultivars (Table 2). Under submerged conditions, the activity of AAO decreased slightly in all the cultivars. After re-aeration, the activity of AAO decreased sharply in FR 13A, while changes in AAO activity in CR 383-10 and IR 42 were negligible. Possibly the tolerant cultivar maintained a higher ascorbic acid content due to lower activities of AAO, which might overcome the deleterious effect of submergence. Ascorbic acid is a common biological antioxidant and its role in counteracting post-anoxic injury in plants has been demonstrated (Crawford and Braendle 1996, Biemelt et al. 1998).

Under submergence, the tolerant cultivar maintained higher dry weight, measured on the basis of per g fresh weight of leaves (Table 3). This showed that the tolerant cultivar was able to maintain greater cell integrity than the other two cultivars during the same period of submergence. After exposure of plants to air there might be some peroxidation of lipids in rice leaves. The product of

Table 1: Changes of catalase (mmol H_2O_2 decomposed min^{-1} g^{-1} FW) and peroxidase (changes in O.D. g^{-1} FW) activities in rice leaves caused by submergence and subsequent re-aeration

	Catalase			Peroxidase		
Cultivar	C	S	R	C	S	R
FR 13A	22.8 ± 2.2	2.85 ± 0.07	4.08 ± 0.13	46.9 ± 2.2	12.3 ± 0.3	14.3 ± 1.1
CR 383-10	21.9 ± 1.3	1.38 ± 0.22	2.27 ± 0.15	33.7 ± 3.8	7.5 ± 1.1	6.3 ± 1.4
IR 42	22.2 ± 1.8	2.50 ± 0.09	2.88 ± 0.11	48.8 ± 1.3	10.6 ± 0.5	6.9 ± 0.6

C = control; S = submerged for 8 days; R = 24 h after start of re-aeration (mean ± S.D.).

Table 2: Changes of superoxide dismutase (units g^{-1} FW) and ascorbic acid oxidase [μmol ascorbic acid decomposed $(30\ min)^{-1}$ g^{-1} FW] activities in rice leaves caused by submergence and subsequent re-aeration

	Superoxide dismutase			Ascorbic acid oxidase		
Cultivar	C	S	R	C	S	R
FR 13A	167 ± 5	67 ± 4	75 ± 5	30.0 ± 1.8	27.0 ± 2.2	8.7 ± 1.5
CR 383-10	127 ± 4	53 ± 2	58 ± 3	45.5 ± 1.8	42.6 ± 1.0	40.8 ± 1.8
IR 42	166 ± 5	64 ± 1	65 ± 2	44.3 ± 3.3	39.5 ± 0.9	39.5 ± 2.1

C = control; S = submerged for 8 days; R = 24 h after start of re-aeration (mean ± S.D.).

Table 3: Changes of dry weight (mg g^{-1} FW of leaves), malondialdehyde (nmol g^{-1} FW) and proline ($\mu g\ g^{-1}$ FW) content in rice leaves caused by submergence and subsequent re-aeration

Cultivar	Dry weight			Malondialdehyde			Proline		
	C	S	R	C	S	R	C	S	R
FR 13A	223.3 ± 1.5	199.7 ± 6.6	185.3 ± 3.0	4.8 ± 0.4	4.5 ± 0.5	6.0 ± 0.4	76.0 ± 1.4	84.0 ± 2.2	66.7 ± 3.5
CR 383-10	235.6 ± 4.0	145.0 ± 8.0	136.3 ± 6.0	6.7 ± 0.5	4.8 ± 0.4	8.9 ± 0.1	70.2 ± 1.8	106.9 ± 7.5	78.2 ± 3.3
IR 42	216.7 ± 11.6	161.0 ± 5.6	152.7 ± 2.1	5.7 ± 1.2	4.2 ± 0.3	9.1 ± 1.0	68.0 ± 4.5	113.8 ± 5.8	85.9 ± 8.0

C = control; S = submerged for 8 days; R = 24 h after start of re-aeration (mean ± S.D.).

lipid peroxidation, i.e. MDA content, was high in the susceptible cultivar IR 42, followed by CR 383-10.

Submergence caused the plants to accumulate proline (Table 3). During air adaptation the content of proline decreased. The accumulation of proline in a wide variety of species under various types of abiotic stresses is well known. Alia and Saradhi (1993) reported that suppression of mitochondrial electron transport was the primary reason for stress-induced proline accumulation in plants. Under submergence, normal growth of mitochondria is affected (Shibasaka and Tsuji 1988), resulting in accumulation of proline. However, the damage appeared to be higher in IR 42 than in CR 383-10 and FR 13A. In addition to being an osmoticum, proline may also act as a sink of energy, a nitrogen storage compound, a scavenger for hydroxyl-radicals and a compatible solute that protects enzymes and cellular structures (Smirnoff and Cumbes 1989). The rate at which proline disappeared after air adaptation suggested that excess proline might be used in adaptation when plants were transferred from hypoxia to normoxia, and the intolerant cultivar used more proline than the tolerant cultivar during the same period of air adaptation.

Reactions involving O_2 free radicals are an intrinsic feature of plant senescence and promote the process of oxidative deterioration that contributes to cell death (del Rio et al. 1998). A number of abiotic stresses, including submergence, lead to overproduction of reactive oxygen intermediates, including H_2O_2, causing extensive damage (Drew 1997). However, the damage could be reduced in those plants that have well-defined systems to protect against the superoxide radical (O_2^-). One protective system involves superoxide dismutase (SOD), converting superoxide radicals to hydrogen peroxide, which is reduced to water by peroxidases or catalases. It has been reported that tolerant species (*Iris pseudacorus*) differ from intolerant species (*I. germanica* and *Glyceria maxima*) in being able to increase SOD activity during the period of anaerobic incubation and thus enter the post-anoxic phase well equipped to counteract the potential hazards of superoxide generation (Monk et al. 1987). In the present investigation, however, it was noted that SOD activity decreased under submergence as compared to aerobically grown plants in both tolerant and intolerant rice cultivars.

In rice, a group of antioxidative enzymes may be involved in submergence tolerance. In addition to SOD, both CAT and PER are also important in

protecting from reactive oxygen damage. The involvement of ascorbic acid appeared to be very important in developing a defense system against post-submergence injury in rice, as the tolerant cultivar exhibited significantly lower AAO activity.

Zusammenfassung

Änderungen antioxydativer Enzyme und Wachstumsparameter in Anhängigkeit von vollständiger Überflutung und darauf folgender Zurücknahme bei Reis-Kultivaren mit unterschiedlicher Überflutungstoleranz

Das Experiment wurde mit drei indischen Reis-Kultivaren (*Oryza sativa* L.), IR 42, CR 383-10 und FR 13A, die empfindlich oder tolerant gegenüber einer Überflutung sind, durchgeführt. Eine schädliche Wirkung der Überflutung wurde sowohl an der oberen Trockenmasse als auch dem Chlorophyllgehalt während auch nach Beendigung der Überflutung festgestellt. Die Reduktion war geringer bei dem toleranten Kultivar. Der tolerante Kultivar FR 13A hatte ein höheres Trockengewicht während und nach der Überflutung und akkumulierte weniger Prolin und Malondialdehyd als die anderen Kultivare. Die Enzymaktivitäten, bestimmt auf der Basis je g Frischgewicht der Blätter zeigten für Katalase, Peroxidase und Superoxiddismutase höhere Werte bei FR 13A sowohl unter Wasser als auch 24 Stunden nach Zurücknahme der Überflutung. Ascorbinsäure und Oxidase Aktivitäten waren bei FR 13A dagegen geringer. Der Kultivar CR 383-10, der eine hohe Kapazität für die Bildung oberirdischer Trockemasse unter normalen Bedingungen aufweist, zeigte einer höhere Längenwachtumsrate unter Überflutung, war aber empfindlich hichsichtlich der Enzymaktivitäten und anderer Parameter gegenüber Überflutung und Zurücknahme der Überflutung wie IR 42.

References

Alia, and P. P. Saradhi, 1993: Suppression in mitochondrial electron transport is the prime cause behind stress induced proline accumulation. Biochem. Biophys. Res. Commun. **193,** 54—58.

Arnon, D. I., 1949: Copper enzymes in isolated chloroplast: polyphenol oxidase in *Buta vulgaris*. Plant Physiol. **24,** 1—15.

Bates, L. S., R. P. Waldren, and L. D. Teare, 1973: Rapid determination of free proline for water-stress studies. Plant Soil **39,** 205—207.

Biemelt, S., U. Keetman, and G. Albrecht, 1998: Re-aeration following hypoxia or anoxia leads to activation of the antioxidative defense system in roots of wheat seedlings. Plant Physiol. **116,** 651—658.

Chance, B., and A. C. Maehly, 1955: Assay of catalases and peroxidases. In: S. P. Colowick, and N. O. Kaplan (eds), Methods in Enzymology, Vol. 2, pp. 764—775. Academic Press, New York.

Chowdhury, S. R., and M. A. Choudhuri, 1985: Hydrogen peroxide metabolism as an index of water stress tolerance in jute. Physiol. Plant. **65,** 503—507.

Crawford, R. M. M., and R. Braendle, 1996: Oxygen deprivation stress in a changing environment. J. Exp. Bot. **47,** 145—159.

Drew, M. C., 1997: Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. Ann. Rev. Plant Physiol. Plant Mol. Biol. **48,** 223—250.

Giannopolitis, C. N., and S. K. Ries, 1977: Superoxide dismutases: occurrence in higher plants. Plant Physiol. **59,** 309—314.

Heath, R. L., and L. Packer, 1968: Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. **125,** 180—198.

Ito, O., E. Ella, and N. Kawano, 1999: Physiological basis of submergence tolerance in rainfed lowland rice ecosystem. Field Crops Res. **64,** 75—90.

Lowry, O. H., N. J. Rosebrough, A. I. Farr, and R. J. Randall, 1951: Protein measurement with the Folin-phenol reagent. J. Biol. Chem. **193,** 634—635.

Monk, L. S., K. V. Fagerstedt, and R. M. M. Crawford, 1987: Superoxide dismutase as an anaerobic polypeptide. A key factor in recovery from oxygen deprivation in *Iris pseudacorus*. Plant Physiol. **85,** 1016—1020.

Olliver, 1967: Ascorbic acid estimation. In: W. H. Sebrell, and R. S. Harris (eds), The Vitamins, pp. 338—359. Academic Press, New York.

del Rio L. A., G. M. Pastori, L. M. Palma, L. M. Sandalio, S. Francisca, J. C. Franscisco, A. Jiménez, E. L. Huertas, and J. A. Hernández, 1998: The activated oxygen role of peroxisomes in senescence. Plant Physiol. **116,** 1195—1200.

Roy, J. K., 1993: Breeding approaches for increasing productivity of rainfed ecosystem. In: K. V. S. R. K. Row (ed.), Proceedings of National Symposium on Advances in Rice Genetics and Breeding, pp. 15—17. Central Rice Research Institute, Cuttack, India.

Sagisaka, S., 1976: The occurrence of peroxide in a perennial plant. Populus Gelrica Plant Physiol. **57,** 308—309.

Sarkar, R. K., and S. K. Bera, 1997: A comparison of the submergence response of elongating and non-elongating flood tolerant deep water rice. Indian Agric. **41,** 299—303.

Sarkar, R. K., R. K. Sahu, and R. N. De, 1998: Tolerance for submergence in rainfed lowland rice under repetition of flooding. Int. Rice Res. Notes **23,** 31—32.

Shibasaka, M., and H. Tsuji, 1988: Respiratory properties of mitochondria from rice seedlings germinated under water, and their changes during air adaptation. Plant Physiol. **86,** 1008—1012.

Smirnoff, N., and Q. J. Cumbes, 1989: Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry **28,** 1057—1060.

Ushimaru, T., M. Shibasaka, and H. Tsuji, 1992: Development of O_2^--detoxification system during air adaptation of submerged rice seedlings. Plant Cell Physiol. **33,** 1065—1071.