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Physiological Changes Associated with Submergence Tolerance in Genetically Diverse Lowland Rice Genotypes

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ABSTRACT. *Rainfed lowland rice ecosystem is affected not only by water deficit but also by excess water leading from partial to complete submergence. Although the rice plant is well adapted to aquatic environment, it is unable to survive if completely submerged in water for several weeks. Damage to rice plants due to excess water has been advocated to occur during submergence and also on entry of oxygen after the recession of flood water. Breeding rice varieties with ample submergence tolerance is one of the approaches to alleviate the adverse effects of submergence which requires incorporation of physiological traits linked to tolerance mechanisms.*

In a controlled pot culture experiment where 21 days old plants of six rice genotypes differing in submergence tolerance were subjected to 10 days complete submergence. Underwater shoot elongation, shoot carbohydrate status and the activity of enzymes catalase and superoxide dismutase (SOD). Submergence tolerant varieties Swarna Sub1, FR13A and NDR 9730018 had higher carbohydrate status and lower shoot elongation during submergence, consequently higher plant survival. Catalase and superoxide dismutase activity before submergence was almost at par in all the genotypes but increased after de-submergence. A time course study of SOD activity indicated that the increase continued until 4 hrs after de-submergence which showed declining trend subsequently until 24 hrs. Submergence tolerant genotypes showed much higher increase in SOD activity as compared to intolerant ones. This can be correlated with higher survival rates observed in tolerant genotypes possibly through mitigating the adverse effects of reactive oxygen species. Low under water shoot elongation, high shoot carbohydrate and higher post submergence SOD activity could be possible physiological markers for screening of rice germplasm for submergence tolerance.

INTRODUCTION

Rice (*Oryza sativa* L.) is the major staple food of millions of the people in the world especially in Asia. National food security stems primarily on the productivity of rice followed by wheat. In India, the annual production of 85.5 million tons of rice with an average productivity of 1.9 t/ha comes from an area of 44.5 million ha spread over several ecologies of which about 17 million ha is rainfed, drought and submergence prone (Singh, 2002). Rainfed lowlands contribute about 19% of the national rice production where

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submergence has been identified as the third important constraint limiting rice production (Hosssain and Laborte, 1996).

Flooding induced submergence results in substantial yield loss in rice which depends upon plant type, crop growth stage and the intensity and duration of submergence. Plant survival during submergence also depends upon light intensity and flood water characteristics namely turbidity, temperature, turbulence and pH. Flooding or submergence is known to create hypoxia or anoxia in the plant system due to 10000 times slower diffusion of gases in water (Armstrong, 1979), which produces several metabolic disturbances leading to tissue damage and eventually death of plants when conditions are too harsh. Submergence induced hypoxia/anoxia in plants shifts entire metabolic pathway from aerobic to anaerobic fermentation which though is most inefficient producing only two adenosine tri phosphate (ATP) molecules but is essential as a source of energy supply either for maintenance process associated with survival or maintenance process and growth (Greenway and Setter, 1996; Setter *et al.,* 1994) hence it is important for plant tolerance to anaerobiosis (Drew, 1990; Alpi and Beevers, 1983). High carbohydrate status prior to submergence and after de-submergence, thus, has important role to play through continued supply of substrate for energy production needed for survival and recovery growth after recession of flood water (Ram *et al*., 2002).

Rice and other aquatic plants tend to elongate when completely submerged and try to emerge on the surface of water through shoot elongation at the expense of reserve substrates used in energy production. In flash flood rice growing areas of eastern India, where submergence occurs only for 1 - 2 weeks, excess shoot elongation is eventually harmful resulting in lodging and death of plants when flood water recedes. The impact of these finding is that elongation growth competes with maintenance process for energy and reduces survival during submergence. Reactive oxygen species including super oxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen are the inevitable by products of cell metabolism when molecular oxygen enters suddenly into the cells after exposure of plants to air during post submergence phase. The elevated levels of these free radicals of oxygen cause membrane damage through lipid peroxidation and protein denaturation if not removed rapidly by oxygen scavenging systems (Ella *et al.,* 2003; Jackson and Ram, 2003) Though oxygen scavenging systems (AOS) are constitutive, they can show higher activity in response to re-exposure to oxygen after a period of anoxia induced by flooding or submergence. In the present study, six tolerant and susceptible rice genotypes were evaluated for underwater shoot elongation, carbohydrate status, constitutive and stress inducible antioxidative defense system in order to ascertain if they are related to submergence tolerance of lowland rice and can be used as markers for future breeding programmes.

MATERIALS AND METHODS

Plant culture and imposition of submergence treatment

Healthy seeds of six rice genotypes namely Swarna Sub-1, Swarna, IR 42, FR 13A, NDR 9730018 and NDR 9930111 were surface sterilized with 1% aqueous solution of sodium hypochlorite for 5 min followed by washing with distilled water. Seeds were soaked in water for 24 hrs and subsequently placed on moist filter paper for sprouting. Sprouted seeds were direct seeded at 1 cm depth in earthen pots (25 cm diameter) filled with 8 kg well pulverized farm soil fertilized with recommended dose of N, P_2O_5 and K_2O (80:40:40 kg/ha). Fifteen replicate pots with three plants in each pot were maintained per treatment under completely randomized design (CRD). On day 21, five plants from each treatment were completely submerged for 10 days duration in an outdoor pond under natural condition. At the end of the submergence period, pots were taken out of the pond and placed in shade for recovery for 24 hrs and then transferred to natural field conditions.

Measurements

The measurements of plant height, shoot carbohydrate and active oxygen scavenging system viz. catalase and superoxide dismutase (SOD) in leaves were measured in triplicate before submergence (BS), just after removal of pots from water pond (AS) and at recovery (7 days after de-submergence). Plant survival was recorded 7 days after termination of submergence. Carbohydrate was extracted with 5 mL 80% ethanol thrice following the method of Yemm and Willis (1954). A 0.5 mL aliquot from the pooled supernatants from each treatment was evaporated to dryness on a water bath and 1 mL distilled water was added to each tube. 4 mL of 0.4% Anthrone reagent (prepared in 80% analytical grade sulphuric acid) was added to each tube and placed on boiling water bath for 10 min upon cooling the colour intensity was measured in a spectrophotometer at 620 nm against reagent blank.

Enzyme analysis

Catalase activity in fresh leaves was measured following the method described by Sinha (1972). 500 mg of fresh leaf samples were extracted in 0.1 M potassium phosphate buffer (pH 7.0) and the extract was centrifuged at 10,000 rpm for 30 min. at 4ºC and the supernatant was used as crude extract for enzyme assay.

The crude enzyme extract was prepared by homogenizing 200 mg fresh leaf samples in 8 mL of extraction buffer and subjected to centrifugation at 10000 rpm. The extraction buffer was prepared by adding 4 mL each of 0.05 M potassium phosphate buffer (pH 7.8) and same buffer of pH 7.0 containing 1% (w/v) poly vinyl pyrolidone (PVP). The enzyme activity was assayed following the method described by Asada *et al.* (1972) based on the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) dye by the enzyme SOD.

RESULTS AND DISCUSSION

Underwater shoot elongation and survival

Survival of six rainfed lowland rice varieties in the present study was negatively correlated (r = 0.96) with underwater shoot elongation (Figure 1). The underpinning theory is that shoot elongation occurs at the cost of hydrocarbons and metabolic energy for maintenance processes. Underwater shoot elongation measured as difference in plant height before submergence and just after removal of plants from submergence varied between 8 - 60%, and more elongation being in susceptible variety IR42. Submergence tolerant varieties had relatively lesser elongation and consequently better survival (Table 1). It is clear from Table 1 that elongation above a threshold level could be detrimental to plants during submergence though survival also depends upon other components and strategies.

Table1. Effects of submergence on under water shoot elongation and survival.

Genotype	Underwater shoot Elongation		Plant Survival	
	Per cent	Angular transformed Value	Per cent	Angular transformed Value
SwarnaSub1	8.2	16.6	100	90.0
Swarna	44.3	41.7	93	74.6
IR42	59.9	50.7	40	39.2
FR13A	16.2	23.7	100	90.0
NDR 9730018	32.7	34.8	100	90.0
NDR 9930111	28.2	32.1	93	74.6
CD 0.01P	-	3.33	-	2.41

Note: 21 day old plants submerged for 10 days, survival recorded after 7 days of termination of submergence.

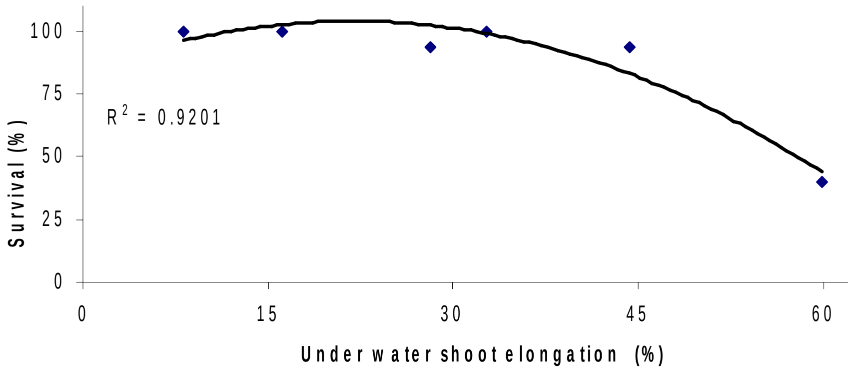

Figure 1. Correlation between under water shoot elongation during submergence and survival of rice plants.

Note: 21 day old plants submerged for 10 day in out door pond under natural conditions.

The importance of slow growth in rainfed lowland rice, unlike deepwater and other aquatic plants, during submergence was suggested to be beneficial which prevents damage due to lodging once the flood water recedes after a short spell of flash flood (Jackson and Ram, 2003; Ram *et al*., 2002; Singh *et al*., 2001). Additional beneficial evidence comes from higher shoot carbohydrate levels observed in the present study, in slow elongating submergence tolerant rice varieties SwarnaSub1 and FR13A (Table 2) which might maintain prolonged energy supply for maintenance processes as suggested by Setter *et al*. (1994) and Greenway and Setter, (1996).

Carbohydrate content and submergence tolerance

Carbohydrate being the prime substrate of energy production has strong relevance to submergence tolerance. Genotypes with higher carbohydrate before and after submergence had better survival during a 10 days complete submergence. Submergence tolerant varieties FR-13A and Swarna Sub1 with 70 and 66% more carbohydrate than susceptible variety IR42 prior to submergence, showed 100 per cent survival against only 40% survival in IR 42 (Table2). Even after de-submergence tolerant varieties maintained higher carbohydrate which enabled them to recover faster than susceptible. FR13A and Swarna Sub1 accumulated 65 and 40% more carbohydrate over a recovery period of only 7 days during which the susceptible variety IR42 could gain only 3 per cent (Table2). A strong positive correlation was observed between carbohydrate level prior to submergence ($r = 0.99$), just after de-submergence ($R^2 = 0.99$) and at recovery ($R^2 = 0.97$) and survival (Figure 2), indicating the importance of carbohydrate in submergence tolerance of rice plants.

Table2. Effect of submergence on shoot carbohydrate status of rice varieties.

Genotype	Shoot Carbohydrate (mg/g/dry weight)		
	BS	**AS**	**AR**
SwarnaSub1	72.6±3.2	42.0±0.3(42.1)*	58.6±0.5(39.5)**
Swarna	58.8±3.6	34.8±1.8(40.8)	48.2±4.3(38.5)
IR42	43.9±0.8	28.9±1.2(34.1)	29.8±0.5(3.1)
FR13A	75.4±1.1	37.6±0.4(50.1)	62.2±1.5(65.4)
NDR 9730018	62.8±3.5	39.2±1.8(37.5)	49.4±0.6(26.0)
NDR 9930111	59.6±0.9	35.2±0.9(40.9)	48.3±2.1(37.2)

Note: 21 days old plants submerged for 10 days in out door pond under natural conditions. All values are means of three replications with ±SEM. BS= before submergence, AS= after de-submergence, AR=at recovery (7 days after termination of submergence.
*Figures in parentheses are per cent decrease over corresponding values before submergence
**Figures in parentheses are per cent increase at recovery over corresponding values just after de-submergence (AS).

Greater mortality of susceptible genotype IR42, therefore, seems to be due to starvation and lower energy supply for maintenance and repair processes of membrane integrity during submergence and recovery phase. Thus, it is concluded that higher amount of carbohydrate in plants prior to submergence and its slower depletion is often positively correlated with the level of submergence tolerance (Santosa *et al.*, 2007; Ram *et al*., 2002; Mallik *et al*., 1995).

Post submergence injury

It is believed that when plants are exposed to air after a period of anoxia, they suffer due to production of highly reactive species of free radicals of oxygen. In our experiments also, we observed that rice plants though appeared softened and droopy just after de-submergence, they did not show sign of mortality. However, during the recovery period of 7 days, a variable degree of leaf drying and mortality was observed in different rice varieties depending upon the level of their submergence tolerance. A progressive drying and subsequent death of plants several days after de-submergence, therefore, indicate the occurrence of a series of events during post submergence period. Anticipating the adverse effects of free radicals of oxygen produced in rice plants during the recovery period, we measured the activity of catalase and superoxide dismutase enzymes (which are known scavengers of free radicals) to ascertain if these enzymes are correlated with the observed genetic variability of rice varieties for submergence tolerance.

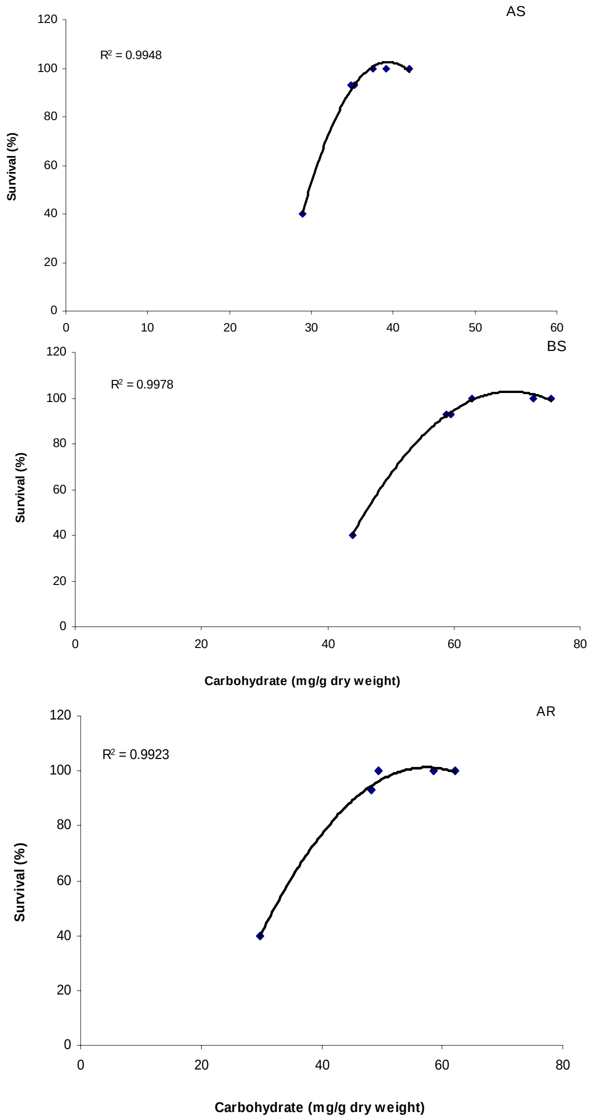

Figure 2. Correlation between carbohydrate content and survival of rice plants

Note: 21 day old plants submerged for 10 day in out door pond under natural conditions. BS: before submergence, AS: after de-submergence, AR: at recovery (7 days after termination of submergence).

The activity of enzyme catalase in 21day old plants of different varieties was almost similar prior to submergence but increased when measured just after removal of plants from submergence. The magnitude of increase was 42 - 58% in tolerant varieties against only 29% in intolerant variety IR42 (Table 3). Similar increase in catalase activity in response to re-exposure of plants to air after a period of anoxia or submergence has been reported in rice varieties different than that used in this experiment (Ushimaru *et al*., 1992 and 1999).

Table 3. Effect of submergence on catalase activity in leaf of rice genotypes.

Genotype	Catalase activity (units/g fresh weight)		Increase (%)
	BS	AS	
SwarnaSub1	476.7±7.2	714.3±17.0	49.8
Swarna	496.9±11.6	707.5±16.7	42.3
IR42	436.9±7.9	564.4±02.1	29.1
FR13A	470.0±2.9	743.6±21.2	58.2
NDR 9730018	485.3±6.8	721.9±13.4	48.8
NDR 9930111	436.9±8.0	651.9±06.2	49.2

Note: 21 day old plants submerged for 10 day in out door pond under natural conditions. BS: before submergence, AS: after de-submergence. Increase (%) = (AS-BS/BS)×100. 1 enzyme unit = increase of 0.01 OD per min under specific assay conditions.

Superoxide dismutase which removes superoxide radicals is another important enzyme associated with anti-oxidative defense system. In the present study SOD activity increased on all the rice genotypes. The increase in activity was about 2 - 3 folds in tolerant varieties but only 1.0 - 1.5 folds in intolerant ones (Figure 3).

Time course measurement revealed that SOD activity increased up to 4 hrs after de-submergence, irrespective of the varieties after which the enzyme activity tends to decline gradually. Even then, the activity was still higher than the initial level in non submerged control plants. The observed time course behaviors of enzyme activity indicate that probably the maximum damaging effect of active oxygen species occurs within 24 hrs of re-exposure to air. Higher enzyme activity seems to be associated with better plants survival and consequently better yield (Table 5). Strong positive correlation was observed between survival and SOD activity after 0, 2, 4, 6 and 24 hrs of de-submergence with R^2 values of 0.99, 0.99, 0.97, 0.98 and 0.93, respectively (Figure 4).

Submergence induced closure of stomata reduces CO_2 availability due to 10^4 folds slow diffusion of gases in water and consequently reduces photosynthesis in stressed plants. However, on sudden entry of oxygen during post submergence phase greatly increases the probability of producing free radicals of oxygen in chloroplast (Marschner, 1995). Accumulation of reduced iron in plants during waterlogging or submergence may facilitate the formation of reactive oxygen species (ROS) through Fe^{2+} catalyzed Haber-Weiss reaction (Hendry and Brocklebank, 1985).

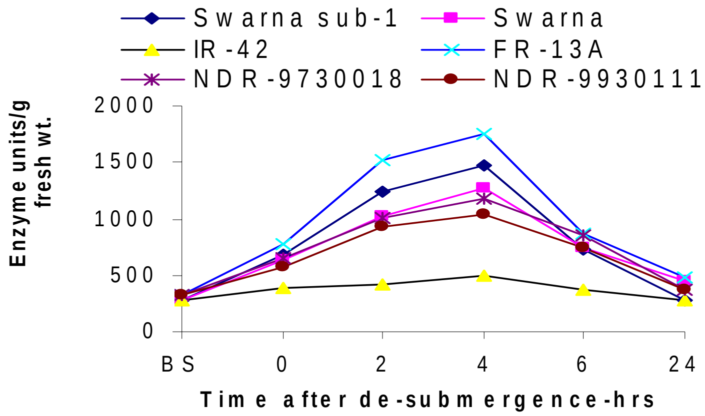

Figure 3. **Effect of complete submergence on time course change in SOD activity during post submergence period in different rice genotypes**

Note: 21 day old plants submerged for 10 days in out door pond. BS: before submergence, 1 unit enzyme = Volume of the enzyme inhibits 50% reduction of NBT under standard assay conditions.

The mechanism of increase in SOD activity in the absence of O_2 during submergence is not clear though it is certain that higher SOD activity during submergence can be advantageous to the plants. Restoration of oxygen supply following a period of submergence induced hypoxia/ anoxia is potentially more damaging than submergence itself. However, the membrane damage measured through online non- destructive emission of ethane (the product of peroxidative damage of membrane) has clearly been shown in rice plants even during submergence and correlated with leaf drying and death (Santosa *et al.*, 2007). Hence, it is concluded that injury to plants from submergence is caused not only during post submergence aerobic conditions but also due to anaerobiosis during submergence (Agarwal and Grover, 2006; Ito *et al.*, 1999). It is also evident that plants with higher constitutive active oxygen scavenging system (AOS) and ability to synthesize them more rapidly and efficiently during post anoxia, presumably suffer less damage (Blokhina *et al.*, 2003) and had better growth during recovery phase (Jackson and Ram, 2003).

Phenology, growth and yield

Submergence in general, delayed 50% flowering in rice genotypes; the maximum delay of 11 days was observed in susceptible genotype IR42 (Table 4). This delay is inevitable since majority of existing leaves and/or shoots die during submergence and the new growth occurs during the recovery phase. Delayed flowering also reduces the grain filling period which directly affects the grain yield as is evident from Table 5. Large variability was also observed in adverse effects of submergence on biomass production in rice genotypes. Submergence tolerant genotypes irrespective of their biomass before submergence showed reduction during submergence, the magnitude of reduction varied between 10 – 35%, the highest being in susceptible genotypes (Table 4). Earlier studies have also indicated that submergence tolerant genotypes accumulate higher dry matter before submergence and maintained enough dry matter after de-submergence to sustain recovery growth. (Singh *et al.*, 2001; Chaturvedi *et al.*, 1995).

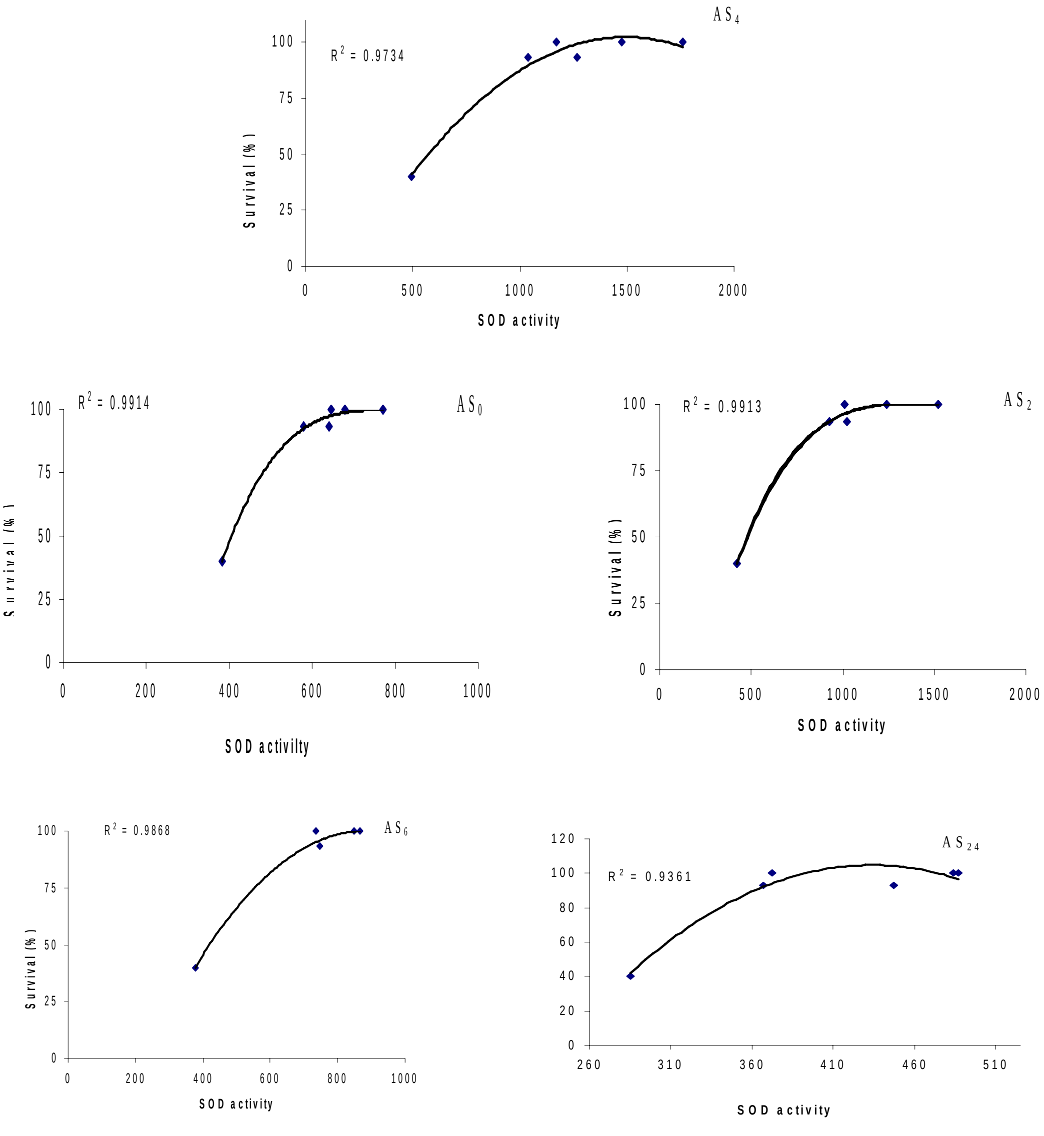

Figure4. Correlation between superoxide dismutase activity during post submergence period and plant survival after 10 days of complete submergence in water.

Note: AS_0: just after de-submergence, AS_2, AS_4, AS_6 and AS_{24} indicate measurements after 2, 4, 6 and 24 hrs after de-submergence.

Table 4. Effect of submergence on days to 50% flowering and shoot biomass at maturity in rice genotypes

Genotype	Days to 50% flowering		Biomass $hill^{-1}$(g)	
	Control	Submerged	Control	Submerged
SwarnaSub1	103± 0.7	107±0.7	62.1±6.5	55.6±8.5(10.4)
Swarna	104± 0.9	108±0.9	76.1±10.5	55.1±8.6 (27.5)
IR42	106± 1.1	117±0.4	53.1±14.7	34.4±5.7 (35.2)
FR13A	110±1.7	114±0.9	62.5±2.6	45.1±17.0 (27.8)
NDR 9730018	105±1.1	109±0.8	59.5±2.9	51.5±5.3 (13.4)
NDR9930111	107±1.6	109±1.1	52.5±6.5	42.2±5.5 (19.6)

Note: 21 day old plants submerged for 10 days in out door pond under natural conditions.
All values are means of three replications with ±SEM. Figures in parentheses are per cent decrease over control (non submerged)

Table 5. Effect of submergence on yield and yield attributes in rice genotypes

Genotype	Panicle weight $hill^{-1}$(g)		Grain yield $hill^{-1}$(g)	
	Control	Submerged	Control	Submerged
SwarnaSub1	27.7±4.7	25.6±4.7(7.5)	24.6±3.4	19.2±3.4 (21.9)
Swarna	30.6±4.7	22.5±3.4 (26.4)	24.9±3.5	15.4±2.0 (38.1)
IR42	16.7±3.2	12.6±7.9 (24.5)	12.8±2.9	04.9±1.2 (61.7)
FR13A	19.4±1.2	14.0±1.1 (27.8)	13.6±1.3	09.1±2.9 (33.1)
NDR 9730018	24.1±11.1	20.7±2.8(14.1)	18.5±9.4	15.05±2.0 (18.6)
NDR9930111	15.8±1.6	14.4±1.6 (8.8)	13.5±4.0	12.3±1.0 (8.8)

Note: 21 day old plants submerged for 10 days in out door pond under natural conditions.
All values are means of three replications with ±SEM. Figures in parentheses are per cent decrease over control (non submerged)

Grain yield was highest in Swarna followed by Swarna Sub-1, NDR 9730018, NDR 9930111, FR 13 A and IR 42 under non submerged control condition whereas under submergence, Swarna Sub 1 had the highest grain yield followed by Swarna, NDR 9730018, NDR 9930111, FR 13 A and IR 42 respectively (Table 5). FR13A though is the world's most submergence tolerant genotype, but has lower yield due to its poor agronomic characteristics, hence, not preferred by the farmers for cultivationss. Another submergence tolerant genotype Swarna Sub 1, recently developed by International Rice Research Institute Philippines, inherits submergence tolerant gene in the background of most popular rice variety Swarna and is the potential variety having maximum grain yield under submergence conditions (Table 5).

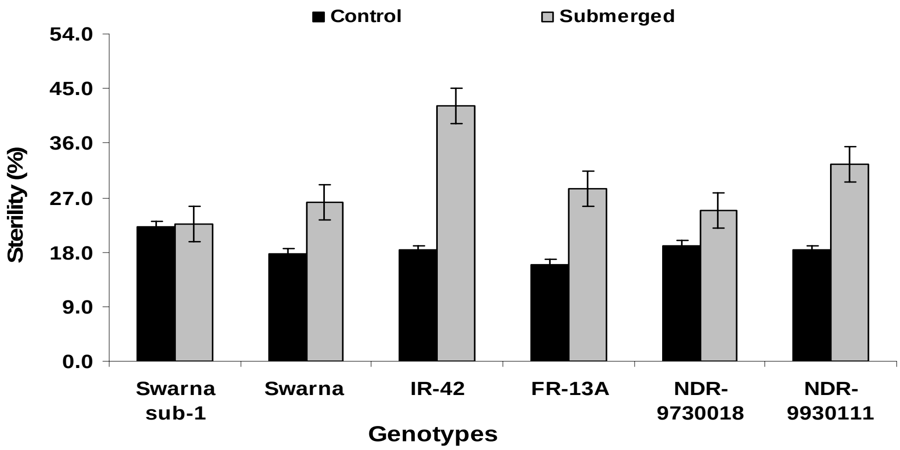

Figure 5. Effect of submergence on sterility (%) in rice genotypes.

Note: 21 day old plants submerged for 10 days in out door pond under natural conditions.

We have also identified two more rice genotypes *viz* NDR9730018 and NDR9930111 which have better grain yield and lower adverse effects of submergence. IR42 had the highest reduction in grain yield under submerged condition primarily due to lowest panicle weight and highest grain sterility (42%). Grain sterility was also more in FR13A and NDR 9930111which seems to be their genetic character (Figure 5). Swarna Sub1 and NDR9730018 had lower grain sterility and better yield under submergence.

FR13A though is a submergence tolerant genotype but had lower grain yield because of its low yield potential and high sterility. However, this genotype has extensively been used as a prime source for introgression of submergence tolerant gene(s) into the poplar high yielding rice varieties.

CONCLUSIONS

In the present study with newly developed submergence tolerant genotypes, we validated the importance of two physiological traits like underwater shoot elongation and carbohydrate status before and after submergence for their association with submergence tolerance. Submergence tolerant genotypes displayed low to moderate under-water shoot elongation and higher carbohydrate status prior to submergence which is closely related to plant survival. Higher carbohydrate levels after de-submergence is also useful in recovery growth during post submergence phase. Genotypes capable of inducing anti oxidative defense systems during hypoxia/anoxia and after re-exposure to air have better submergence tolerance measured as higher plant survival and grain yield. It is evident that underwater shoot elongation and carbohydrate status could possibly be used as physiological markers for rapid screening large number of rice gemplasm for submergence tolerance.

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