https://doi.org/10.56093/ijas.v90i12.110339

Indian Journal of Agricultural Sciences **90** (12): 2351–6, December 2020/Article

Variation in aerobic recovery and vigor of cultivated and wild rice genotypes after anaerobic germination stress in rice (*Oryza sativa*)

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Received: 31 December 2019; Accepted: 16 March 2020

ABSTRACT

Hypoxia due to flooding during germination is a common abiotic stress for direct sown rice (DSR). There are available protocols for evaluating tolerance to anaerobic germination including seedling survival percentage and coleoptile elongation which is helpful in surviving shallow flooding. Here we studied previously underexplored aerobic recovery after extended anaerobic stress during germination and seedling vigor after recovery. A procedure was developed for screening rice (*Oryza sativa* L.) genotypes for measuring aerobic recovery and vigor of recovery and demonstrated in a set of 20 cultivars and 30 wild rice accessions. Six wild rice accessions (NKSWR52, NKSWR3, NKSWR57, NKSWR226, NKSWR98 and NKSWR83) and cultivar Pokkali showed the highest recovery percentage and three wild rice accessions (NKSWR 97, NKSWR 57 and NKSWR 226) and cultivar Apo showed the most vigorous recovery (grew >4.5 cm in 7 days). The linear correlation analysis of different seed parameters show that there is a high correlation between percentage of aerobic recovery and vigor of recovery of seedlings and low correlation between anaerobic coleoptile elongation and stored carbohydrates in the seeds. This study also suggested that the mechanism behind recovery of rice seedlings from extended hypoxia during germination is due to ability to stay alive by slowing down metabolism and entering to a dormant state.

Key words: Aerobic recovery, Anaerobic germination stress, Rice, Vigor

Rice (*Oryza sativa* L.) is the staple food for half of the world population and is cultivated in 65 M ha with global production of 503.9 M tonnes (FAO 2017). To feed the growing population and ensure food security under changing climate, it is important to sustain and improve rice productivity. In Asia, where most of the world's rice is grown and consumed, about 20 M ha of rice growing area is prone to flooding. In India and Bangladesh alone, more than 5 M ha of rice fields are flooded during some period of their growth (IRRI 2014). Heavy monsoon rain leads to extended flooding in major rice growing areas of India, especially in the eastern states where it remains the third most serious reason for yield losses. Flooding during early stage of rice germination may lead to entire crop loss, particularly in DSR cultivation (Jackson and Ram 2003). The duration of waterlogging during germination directly affects germination percentage, plant stand and growth rate considerably. Rice is the only cereal crop that can be cultivated under flooded ecosystem. Some rice cultivars can germinate well under water under anaerobic conditions but most are sensitive to prolonged hypoxia (Angaji *et al.* 2010).

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In many rice varieties, submergence tolerance is contributed by Sub1A gene coding for an ethylene response factor (Xu *et al* 2006, Fukao *et al.* 2006). However, tolerance to anaerobic germination does not involve the Sub1A locus as rice cultivars without Sub1A locus, namely, M202 and Nipponbare, displayed good anaerobic germination tolerance (Xu *et al.* 2006). Rice plants can tolerate anaerobic conditions caused by water stagnation by adopting mainly two strategies, either elongating the stem, thus escaping the stress, or remaining dormant under water until water recedes (Luo *et al.* 2011, Singh *et al.* 2017). However these adaptive mechanisms do not help at time of germination and early seedling growth. In hypoxia condition during germination, rice behaves differently than other cereals by suppressing root growth and elongating the coleoptile (Tsuji 1973; Alpi and Beevers 1983). If the coleoptile cannot reach out for air in a few days, the seedling will perish due to oxygen starvation. Previous studies have extensively worked on anaerobic germination survival percentage and coleoptile elongation, and mapped QTLs for the trait and identified trehalose 6-phosphate phosphatase 7 (*TPP7*) gene responsible for rapid coleoptile elongation (Angaji *et al.* 2010, Septiningsih *et al.* 2013, Kretzschmar *et al.* 2015). But neither of these traits can help survival and recovery of rice seedlings after prolonged flooding.

If anaerobically germinated rice seedlings can remain alive during prolonged flooding and recover vigorously when flood recedes it will help avoid crop failure. Hence present study was designed to assess the capability of anaerobically germinated rice seedlings to recover from prolonged flooding and vigor of the recovered seedlings. We developed a high throughput screening protocol for screening the recovery of anaerobically germinated rice seedlings and screened 20 cultivars and 30 wild rice accessions to identify the most tolerant genotypes.

MATERIALS AND METHODS

Study location, planting materials used: The screening was conducted in the phenotyping facility of ICAR-NIPB, New Delhi with 30 wild rice accessions and 20 cultivars (Table 1) during *kharif* season of 2018 and 2019. The wild rice accessions were part of a collection available in our laboratory (Singh *et al.* 2018).

Screening for aerobic recovery of anaerobic germinated rice: A new protocol was developed for screening of rice genotypes for tolerance to anaerobic germination and recovery after hypoxia. 50 ml falcon tubes (11.5 cm height and 3 cm diameter) were used for experiments. 5ml of 0.8% agarose was added to each 50 ml tubes and allowed to solidify. For each genotype, 15 healthy seeds were placed

Table 1 Performance of fifty rice genotypes after 18 days of germination and growth under anaerobic in water, percentage recovery from anaerobic stress (Anaerobic recovery percentage: HS- highly sensitive 0-20%,S-sensitive 20-40%, MS – moderately sensitive 40-60%,MT- moderately tolerant 60-80%,T- tolerant 80-90% and HT – highly tolerant >90%),7 days of recovery in air on moist tissue paper (Vigor of recovery - weak >1cm, MW- moderately weak 1-2 cm, MV – moderately vigorous 2-3 cm, V – vigorous 3-4.5 cm and HV – highly vigorous- >4.5cm) 1000 seed weight

Genotype	1000 seed weight (g)	Coleoptile length 18 days after sowing in water (cm)	Aerobic recovery % (after 18 days of anaerobic stress)	Height of recovered seedlings in air for 7 days (cm)
ADT 39	13.9	2.9	60 (MT)	0.7 (W)
Apo	14.90	2.9	84.47 (T)	4.5 (HV)
CR 1009	17.67	3.1	73.33 (MT)	2.3 (MV)
FR 13A	19.33	2.9	15.53 (HS)	0.8 (W)
IR 64	23.17	1.8	42.2 (MS)	1.4 (MW)
IR64-sub1	16.40	1.8	51.13 (MS)	2.1 (MV)
MTU 1010	20.27	2.0	45.07 (MS)	0.8 (W)
Nagina 22	19.87	0.0	0 (HS)	0 (W)
P114	13.83	1.7	57.8 (MS)	1.3 (MW)
P44	15.87	1.9	0 (HS)	0 (W)
Pokkali	25.37	2.5	91.13 (HT)	2.7 (MV)
Pooja	13.67	1.4	20 (S)	0.8 (W)

Contd.

Table 2. (*Concluded*)

Genotype	1000 seed weight (g)	Coleoptile length 18 days after sowing in water (cm)	Aerobic recovery % (after 18 days of anaerobic stress)	Height of recovered seedlings in air for 7 days (cm)
Prabhat	14.77	1.5	22.2 (S)	0.4 (W)
Pusa basmati 1509	27.50	2.8	33.33 (S)	1.7 (MW)
Raj masoori	14.50	2.6	20 (S)	1.5 (MW)
Rajsree	15.77	2.9	48.87 (MS)	3.2 (V)
Ranjeet	22.93	2.8	26.67 (S)	0.4 (W)
Sarjoo 52	21.50	3.2	20 (S)	0.9 (W)
Swarna	16.53	3.8	17.8 (HS)	0.5 (W)
Swarna-Sub1	14.50	3.3	22.2 (S)	0.6 (W)
NKSWR100	22.20	4.2	40 (MS)	0.92 (W)
NKSWR179	22.60	2.6	51.13 (MS)	1.1 (MW)
NKSWR184	24.47	3.9	26.67 (S)	1.4 (MW)
NKSWR185	21.70	1.7	33.33 (S)	1.5 (MW)
NKSWR226	18.53	2.2	95.53 (HT)	5.8 (HV)
NKSWR279	19.77	2.8	31.13 (S)	1.5 (MW)
NKSWR280	21.47	1.9	66.67 (MT)	3.3 (V)
NKSWR283	23.10	3.5	53.33 (MS)	3.2 (V)
NKSWR397	23.60	4.2	75.53 (MT)	3.0 (V)
NKSWR3	24.67	3.7	95.53 (HT)	3.6 (V)
NKSWR30	18.77	2.8	45.07 (MS)	2.3 (MV)
NKSWR51	19.03	2.8	84.47 (T)	3.1 (V)
NKSWR52	19.27	3.4	95.53 (HT)	3.4 (V)
NKSWR53	16.67	4.3	86.67 (T)	2.5 (MV)
NKSWR55	27.17	3.2	33.33 (S)	1.3 (MW)
NKSWR57	25.37	2.7	95.53 (HT)	5.8 (HV)
NKSWR62	19.67	2.3	33.33 (S)	1.3 (MW)
NKSWR68	26.63	3.2	8.87 (HS)	1.1 (MW)
NKSWR69	21.03	3.8	6.67 (HS)	1.5 (MW)
NKSWR70	14.70	2.2	62.2 (MT)	1.6 (MW)
NKSWR72	19.83	1.9	22.2 (S)	2.6 (MV)
NKSWR75	14.87	2.8	55.53 (MS)	1.5 (MW)
NKSWR80	20.40	3.8	24.47 (S)	0.7 (W)
NKSWR83	19.97	3.2	100 (HT)	2.8 (MV)
NKSWR86	20.30	4.5	17.8 (HS)	0.9 (W)
NKSWR88	19.50	2.7	33.33 (S)	2.2 (MV)
NKSWR89	12.67	1.8	51.13 (MS)	1.5 (MW)
NKSWR97	24.17	3.2	66.67 (MT)	5.3 (HV)
NKSWR98	22.67	3.3	97.8 (HT)	1.9 (MW)
NKSWR99	20.97	1.8	31.13 (S)	0.5 (W)
Mean	28.18	19.76	48.41	1.96
SD	7.89	3.89	28.17	1.43
CV (%)	28.01	19.69	58.19	73.06

in three replicates and the seeds were placed top of 5 ml solidified agarose in tubes along the edges and 0.5 ml of 0.8% lukewarm agarose was added to the tubes and allowed to solidify to keep the seeds in place and slightly embedded. The tubes were filled with de-ionized water to ensure complete submergence of seeds under 10 cm depth. The top of each tube was covered with paraffin film to restrict aeration. These tubes were kept at 27^o C for 18 days in the glass house and then coleoptile was measured.

After measurement of coleoptile length, the germinated seeds were kept on moist tissue papers in petri dishes and allowed to grow. Water was added to tissue papers at regular intervals to maintain moisture. The number of seedlings recovered from anaerobic stress was counted and height of the seedlings was measured in centimeter on the seventh day after exposure to air. Statistical analysis, including analysis of variance and pair wise correlation coefficient were estimated for germination percentage, anaerobic coleoptile length, 1000 seed weight and recovery from anaerobic germination stress. Recovery percentage of the lines was estimated using the formula given below

Recovery percentage = (number of recovered seedlings from hypoxia/total number of germinated seedlings) × 100

RESULTS AND DISCUSSION

Variation in coleoptile elongation of rice genotypes under anaerobic germination and percentage of aerobic recovery

For developing a screening protocol to study the ability of rice seedling to recover from anaerobic stress at germination, it is necessary to determine the maximum duration for which anaerobically germinated rice seedlings can remain alive and actively grow under water. Hence, 15 seeds from 4 varieties, IR 64, Pusa Basmati 1509, Pooja and Pokkali, adapted to different rice ecosystems were chosen for initial screening.

To determine the active growth period of coleoptile under hypoxia (under water), 10 seeds were placed in 50 ml falcon tubes on 5ml of 0.8% solidified agarose along the edges of the tube and pouring 0.5 ml lukewarm agarose to fix them. Each genotype was sown with three independent replicates and length of coleoptile was measured between 2nd to 12th day at 2 days intervals. The average coleoptile length measured was plotted as a growth curve (Fig 1A). The rice seeds germinated and coleoptile emerged after1-2 days in all four varieties and they showed exponential growth till 8th day reaching their maximum length around 10th to 12th days (Fig 1A). From the growth curve, it was also evident that the growth pattern and maximum coleoptile length (11.2 to 33.3 cm) differed significantly among genotypes (Fig 1B).

The anaerobically germinated seedlings were taken out on 12th, 15th, 18th and 21st day and placed on a sterile petri dish on wet tissue paper and number of recovered seedlings were counted on the 7th day after placing in petri dish. The recovery percentage of each variety under aerobic conditions gradually declined the period of from 12th to 21st days at different levels (Fig 2). Pokkali, a variety grown in deep water conditions could recover better with 53.33% of the seedlings surviving even after 21 days of anaerobic stress while Pooja, a sensitive variety, declined by 50% after 15 days of anaerobic stress. While aerobic recovery percentage of all varieties declined sharply 15th to 18th day of hypoxia, Pokkali a tolerant genotype showed decline in recovery only after 18 days of hypoxia. Thus, for screening the recovery percentage from anaerobic stress and identifying the most tolerant material anaerobic stress of 18 days is meaningful.

Screening protocol for percent recovery after anaerobic germination stress

Ten healthy seeds of each genotype were taken for screening in three independent replicates. Surface sterilization was not done because it will expose the seeds to air after water imbibition. **S**terile transparent graduated

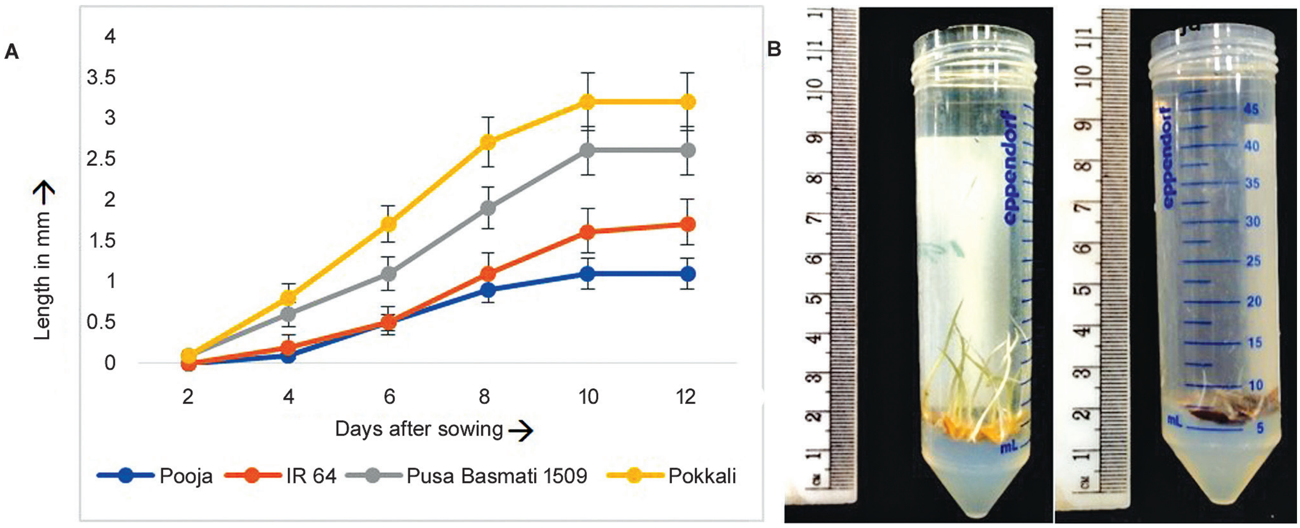

Fig 1 A. Coleoptile elongation of rice cultivars under water in 50 ml falcon tubes between 2 to 12 days after sowing, B. Coleoptile length 12th day of anaerobic germination of contrasting cultivars Pusa Basmati 1509, IR 64, Pokkali and Pooja.

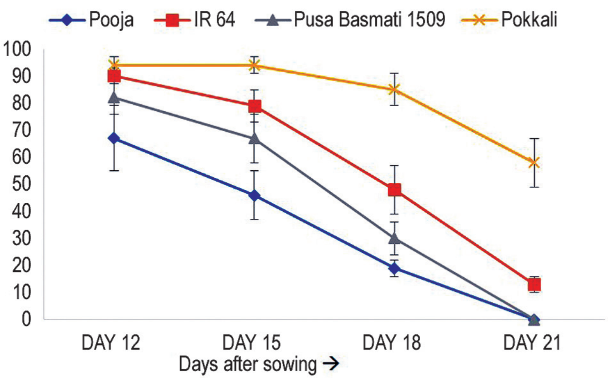

Fig 2 Aerobic recovery percentages of anaerobic germinated seedlings in petri dishes with moist tissue paper after removal from water 12th, 15th, 18th and 21st days after sowing.

50 ml falcon tubes were used as containers for germination as they consumeless space and allow recording observation on coleoptile elongation from outside and hence suitable for large scale screening. Agarose (0.8%) 5 ml was used as growth medium for germination as it is inert and to immobilize the seeds in the tube, 0.5 ml of lukewarm agarose was added to the top of the seeds to make the seeds slightly embedded in agarose. For stress treatment, deionized water was filled in the tubes (10 cm water column). The set-up was kept in a glass house at 27^0 C for 18 days. The tubes were regularly monitored to maintain water depth and check for algal growth and fungal infections. After 18 days of anaerobic treatment, water was decanted and the seedlings were taken out carefully without disturbing the germinated seeds for observations and their coleoptile length was measured. Germination percentage was also calculated by counting the number of seedlings growing under anaerobic conditions. The seedlings removed from each tubes were placed on wet tissue paper in individual petri dishes. On the 7^{th} day of aerobic recovery, seedlings vigor was recorded by measuring the shoot length of recovered seedling in centimeters.

Variation in aerobic recovery of genotypes after anaerobic germination stress

Out of the 50 genotypes screened, 49 geminated under water except Nagina 22 which failed to germinate. The coleoptile length after 18 days of germination and growth under water ranged from 13.6 mm for Pooja to 38.2 mm for Swarna among the cultivars, and from 17.4 mm for NKSWR89 to 44.7mm for NKSWR86 among the wild rice accessions. Average coleoptile length for cultivars was 25.10 mm with standard deviation of 6.66 and for wild rice accessions it was 30.14 mm with SD of 7.99 (Table 2). Though a wider range of variation is expected, wild rice accessions showed better average anaerobic coleoptile elongation, average recovery percentage and average vigor aerobic recovery (Table 1). Interestingly, Swarna showed almost similar performance as Swarna-Sub1 (38.24 mm vs 32.54 mm) while IR 64 and IR 64-Sub1 showed similar performances (18.32 and 18.21 mm). This clearly demonstrated that Sub1 locus had no role on anaerobic germination ability.

Table 2 Variation in component traits of anaerobic germination stress tolerance in wild rice accessions and rice cultivars

Genotype	Parameter	1000 seed weight (g)	Anaerobic coleoptile length (mm)	Aerobic recovery %	Length of recovered seedlings (cm)
Wild rice	Mean	20.86	30.14	54.02	2.30
	SD	3.34	7.99	28.90	1.40
	CV(%)	16.01	26.52	53.49	60.90
Cultivar	Mean	18.11	25.10	39.56	1.41
	SD	4.06	6.66	24.49	1.10
	CV(%)	22.41	26.52	61.90	77.76

Since the recovery from anaerobic conditions ranged from 0 (Pusa 44) to 100 % (NKSWR83), the screened rice genotypes were classified into six groups, namely, highly tolerant (above 90%), tolerant (80-90%), moderately tolerant (60-80%), moderately susceptible (40-60%), susceptible (20-40%) and highly susceptible (0-20%). Only one cultivar each was represented in the highly tolerant group (Pokkali: 91.13%) and tolerant class (Apo: 84.47%) (Table 1). All the other anaerobic germination tolerant genotypes showing better recovery were WR (Wild rice) accessions with NKSWR52, NKSWR3, NKSWR57, NKSWR226, NKSWR98 and NKSWR83 in highly tolerant and NKSWR51 and NKSWR53 in tolerant class (Table1). Among the 9 highly susceptible lines, 3 were WR accessions (NKSWR86, NKSWR69 and NKSWR52) and the rest 6 were cultivars (Pusa 44, FR 13A, Swarna, Pooja, Raj Masoori and Sarjoo 52). IR64, another important cultivar which is previously reported to show a lower anaerobic survival percentage (Angaji *et al.* 2010) and slow coleoptile elongation at early stages of germination (Hsu *et al.* 2015) was found only moderately susceptible and had an aerobic recovery percentage of 42.2%. Pokkali, a traditional cultivar suited to deep water conditions might have better mechanism for tolerance to anaerobic germination. The submergence tolerant variety FR13A from which the *SUB1* QTL for submergence tolerance was identified, though it had moderate coleoptile elongation but it performed poorly during aerobic recovery (15.53% recovery percentage). Previous studies also reported it as susceptible to anaerobic germination stress (Xu *et al.* 2006, Fukao *et al.* 2006). Further, IR64 (42.20%) and Swarna (17.8%) had almost similar aerobic recovery percentage to their Sub1 counterparts IR64 Sub1 (51.13%) and Swarna Sub1 (22.2%) which reinforced that anaerobic germination tolerance has little to do with Sub1 locus (Table 1).

Based on the vigor of recovered seedlings, they were classified into five different groups as weak (0-1 cm), moderately weak (1-2 cm), moderately vigorous (2-3), vigorous (3-4.5 cm) and highly vigorous (>4.5 cm) according to the variations in seedling height. Most of the recovered genotypes (30 genotypes) reached less than 2cm height which are considered as weak or moderately weak. Three wild rice lines (NKSWR97, NKSWR57 and NKSWR226) and one cultivar (Apo) that could grow >4.5 cm in 7 days were the most vigorous. In vigorous category (3 to 3.5 cm) there were 7 genotypes which included one cultivar (Rajsree) and remaining were wild rice accessions (NKSWR280, NKSWR283 NKSWR397, NKSWR3, NKSWR51 and NKSWR52)(Table 1).

The line with highest germination percentage, NKSWR 83 (100%), had an average seedling height of 3.21 cm and the cultivar with highest germination percentage Pokkali (92.2%) had an average seedling height of 2.68 cm which was only moderate. This means aerobic recovery percentage and vigor of recovery is not perfectly correlated. So,it is important that both recovery percentage and seedling vigor during recovery should be considered together while selecting plants with best tolerance for breeding purposes, as it is important that the seedlings should grow faster along with high aerobic recovery percentage.

Correlations among traits used for evaluation of tolerance to anaerobic germination

As the seed weight increases, content of carbohydrate reserves in the rice endosperm also increases. This is the reason why seed weight was included as one of the parameters for evaluating the anaerobic germination tolerance. Linear correlation was significant ($P<0.05$) and positive between coleoptile height and 1000 seed weight (Table 1). The low magnitude of correlation suggests that multiple factors may be involved in determining coleoptile elongation under anoxia and stored carbohydrate content may be one among them. The correlation between aerobic recovery percent and final height of seedlings after recovery was highly significant and positive.

Under anaerobic conditions rice seeds do germinate and grow but shows contrasting effects on shoot and root primordial growth with elongation of coleoptiles without differentiation into leaves and suppression of root growth. Rapid and vigorous elongation of coleoptile ensures survival of rice seedlings under water at the time of germination under shallow flood. In our screening some wild rice accession's coleoptile could elongate as long as 4.45 cm.Correlation analyses showed that the recovery from anaerobic stress had no role (neither cause nor effect) on anaerobic coleoptile elongation. So there is no relationship between how vigorously coleoptiles grow anaerobically and their aerobic recovery percentage.

If germinating rice seeds have the ability to remain alive for longer period of time under water, it will have the ability to successfully tolerate and recover from anaerobic stress. This property can arise either by maintaining anaerobic respiration by using the stored carbohydrates present in the seeds or by arresting the metabolisms and going into a dormant stage. If the seedling can remain alive by maintaining anaerobic fermentation, there will be a correlation between total carbohydrate content in seeds and recovery from anaerobic stress due to availability of more energy source to maintain anaerobic respiration. So after the depletion of available carbohydrates under anaerobic fermentation, the seedlings will die. For checking this hypothesis, the correlation between 1000 seed weight and recovery percentage was studied which revealed that there is hardly any correlation between the two (Table 3). Vigor of recovered seedlings from anaerobic stress also had no correlation with 1000 seed weight or coleoptile elongation under anaerobic conditions (which uses a part of carbohydrate stored in seed). This shows that the ability to remain alive for longer period of time under water and how vigorously recover from the anaerobic condition is independent of amount of carbohydrate present. So it is more likely that the ability to reduce metabolism and entering dormant state is a possible mechanism of survivability of rice seedlings under flooding. Further, if the emerging

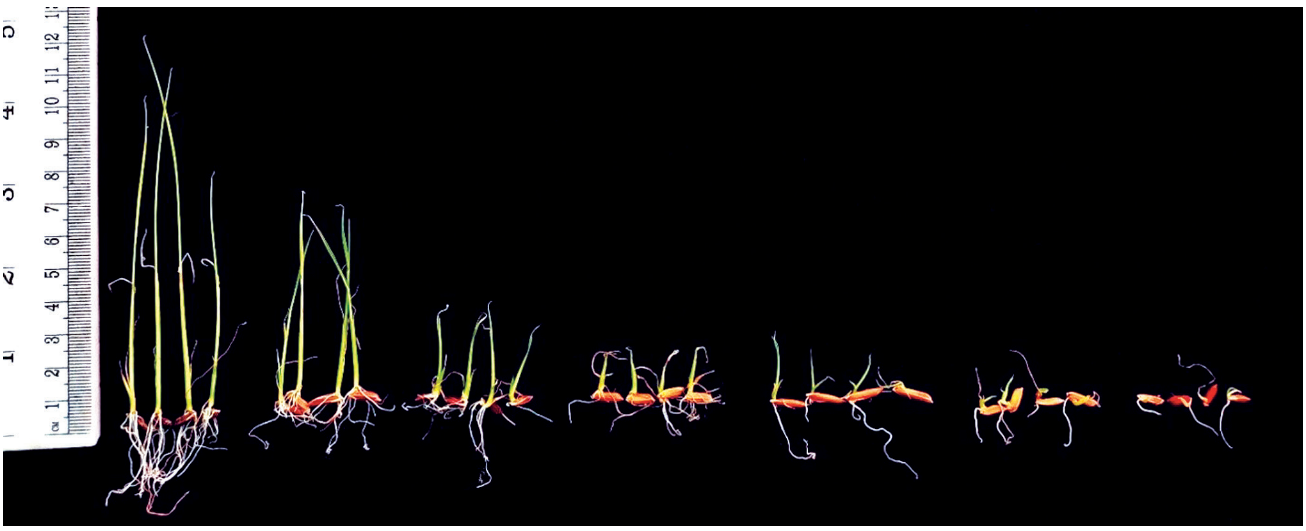

Fig 3 Variations in vigor of recovery of anaerobically germinated seedlings. The coleoptiles of vigorously recovered seedlings are alive and coleoptiles of weakest lines are partially or completely dead.

Table 3 Pearson's correlation coefficients among the parameters used for studying anaerobic germination stress tolerance in rice (ns – not significant * - significant at p = 0.01, **- significant at p = 0.05)

	Coleoptile length	1000 seed weight	Percent recovery	Seedling length
Coleoptile length	1			
1000 seed weight	0.325 *	1		
Percent recovery	0.109^{ns}	0.077^{ns}	1	
Seedling length	0.086^{ns}	0.2^{ns}	0.735**	1

seedlings become dormant, the seedlings can recover once aerobic conditions are restored even if their coleoptiles are dead as the partially germinated embryo would have been still alive along with active meristems. From our experiment, it could be clearly seen that the survived seedlings of the weakest genotypes, though all coleoptiles were dead, still growth could be recovered (Fig 3). Since the embryo alone survived the anaerobic stress the most likely mechanism of survival is by going into a dormant state.

Interestingly percent aerobic recovery had a relatively high correlation to the vigor of recovered seedling with a positive correlation coefficient of 0.735 (Table 3). There is a high chance that the ability of germinated seedling to go dormant after reaching maximum coleoptile height under anaerobic germination could be the main reason for both higher recovery percentage and higher seedling vigor. Also in the screened sample during aerobic recovery, it was observed that the seedlings with long and live coleoptile can easily recover and grow more vigorously (Fig 3). In weakly developing seedlings coleoptile were already dead partially or completely. It showed that if the coleoptile is completely alive after long term exposure to anaerobic stress then the seedling can easily recover and can grow vigorously. But if the coleoptile is partially or completely dead then the recovery is slow and the recovered seedlings grow weak. It showed the seedlings with healthy alive coleoptile would have an advantage of fast response on exposure to air and recover vigorously by coleoptile directly under going photo morphogenesis, emerge leaves and start photosynthesis much quickly.

In the present study we analyzed aerobic recovery percentage and vigor of recovery after germination and growth of rice seedlings under anaerobic stress. For this a screening protocol was developed for measuring the recovery and vigor and screened 20 cultivars and 30 wild rice accessions to identify tolerant and susceptible genotypes, which can be used in future breeding programs. Our results suggest Sub1A locus is not involved in percentage of recovery from anaerobic stress and possible mechanism of tolerance to anaerobic germination tolerance may involve slowing down metabolism and going into a dormant state after initial anaerobic coleoptile elongation under hypoxia, which allows them to survive long duration of low oxygen conditions.

ACKNOWLEDGEMENTS

We are thankful to ICAR-IARI and ICAR-NIPB for providing glasshouse and lab facilities and ICAR-National Professor BP Pal Chair project for providing assistance. We are also thankful to DBT for providing fellowship.

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