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Evaluation of Germination Ability in Rice Seeds under Anaerobic Conditions by Cluster Analysis

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ABSTRACT

Slow seed germination and delayed seedling establishment will become a major problem for rice production in flood-prone lowland areas as sowing method shifts from transplanting to direct seeding. To address this problem, the relationships between genotypic differences in the pattern of water uptake over different times of seed imbibition and adaption to anaerobic conditions were studied. The pattern of water uptake and germination time under aerobic and anaerobic conditions was evaluated for fifty-eight contrasting rice genotypes. Among them, nine lines had been developed for anaerobic germination (AG) and submergence tolerance (Sub1) by the International Rice Research Institute. In addition FR13A, with a designated Sub1 gene and IR42 without the Sub1 gene were used as model genotypes. Results showed that cluster analysis separated the genotypes into three groups based on the water content in seeds at different times of seed imbibition. Most AG+Sub1 lines were placed in cluster 3 which was characterized by rapid water uptake during the first 48 h of seed imbibition and rapid germination under aerobic and anaerobic conditions. Cluster 1 and 2 included genotypes with slower water uptake during the first 48 h and between 96 and 120 h of seed imbibition compared with cluster 3 under anaerobic condition. Therefore, these genotypes germinated more slowly than AG+Sub1 lines. The significant variation between two model genotypes in germination time was not significant under anaerobic condition. The present results verified that the regulation of water uptake during rice seed imbibition play an important rule in germination ability of rice seeds under anaerobic conditions.

Key words: AG+Sub1 lines, flooding-prone areas, germination time, seed imbibition, water uptake rate

INTRODUCTION

Flood-prone lowland areas have been used for the extensive cultivation of rice in Asia, whereas in African regions similar areas have been kept uncultivated despite their suitability for rice cropping. One of major problems for rice production in this area is poor germination and seedling establishment with direct seeding methods due to unpredictable flooding events. Although rice plants are known to be adapted to flood conditions, many rice cultivars are sensitive to low oxygen levels during the germination period which is frequently caused by flooding (Yamauchi *et al*., 1994). Furthermore, although direct seeding is advantageous over transplanting

in terms of labor requirement (Tuong *et al.*, 2000), many prime quality and high yielding rice cultivars are not good performers in direct seeding, resulting in slow development of direct seeding technology in flood-prone area. For its wider cultivation in flood-prone areas, rice cultivars are required to have tolerance to low oxygen levels during germination and adaptability to direct seeding.

Enhancement in the genotypic tolerance to anaerobic conditions during germination is much more inexpensive for poor farmers in developing countries and is more feasible for adoption on a larger scale than other management practices (e.g., coating seeds with calcium peroxide which supplies oxygen to the rice seeds under flooding conditions). Unfortunately, very limited success has been achieved from previous efforts to improve the tolerance of genotypes for anaerobic conditions during germination (Jiang *et al.*, 2004). For instance, Angaji *et al.* (2009) reported that tolerance to flooding during germination seems relatively rare in rice. After screening over 8000 gene bank accessions, elite breeding lines and genotypes, they identified few genotypes with greater ability to germinate under flooding condition; only 0.23% of all accessions were identified with a reasonably high level of tolerance in the initial screening. Subsequent evaluation in replicated trials reduced this number even further i.e., 0.06% (Jiang *et al.*, 2006). This implies that the availability of suitable genetic donors will be a key to introduce tolerance to anaerobic condition during germination into elite cultivars and popular genotypes. Therefore, it is important to conduct more studies into the genotypic variation and function of anaerobic tolerance in rice seeds.

Because low O_2 levels enhance coleoptile elongation during the germination of rice seeds (Perata and Alpi, 1993; Redona and Mackill, 1996), several investigators have taken this as evidence and tried to relate coleoptile elongation or biochemical changes that are initiated by water uptake with tolerance to anaerobic conditions. To our knowledge, however, few results have been reported on the involvement of genotypic variation in water uptake pattern over time in relation to adaption to anaerobic conditions during germination. According to Bewely and Black (1994), water uptake by the seeds follows a triphasic pattern. Phase I represents rapid water uptake, followed by a plateau phase (phase II) and then a post-germination phase of water uptake (phase III). While the use of these phases is convenient to illustrate the events that takes place during germination, it is not yet known whether or to what extent, the genotypic variation in regulation of water uptake during these phases are associated with tolerance to anaerobic conditions during germination.

Many reports have shown that the activities of biochemical changes in seed during germination which are essential for radicle protrusion, are associated with the water uptake pattern on one side and the water uptake patterns with three phases, on the other side, are affected by many factors such as seed structure, physiological potential, germination conditions and genotypes (Kader and Jutzi, 2002; Boyd and Van Acker, 2004; Mandal *et al.*, 2008; Cho, 2010). For example, in most legumes crop, black-colored seeds imbibe more rapidly than white-colored seeds and then germinate earlier (Liu *et al.*, 2007). In snap bean, seed color was associated with water absorption properties (Balkaya and Odabas, 2002). Rapeseed genotypes with yellow seeds showed a faster water uptake than the red or black seeds which led to imbibition damage and lower flooding tolerance (Zhang *et al.*, 2008). Regarding the relationship between water uptake patterns and biochemical changes during seed germination, Yang *et al.* (2007) reported that the degradation of storage proteins mainly happened in the late stage of germination phase II (48 h imbibition) while that of seed maturation and desiccation proteins occurred at the early stage of phase II (24 h imbibition) when seeds were imbibed at 26°C. Thus, we hypothesized that the water uptake pattern during

seed imbibition will differ among rice genotypes, and these differences will play an important role in the tolerance of these genotypes to anaerobic conditions during germination through effects on germination time.

Recently, The International Rice Research Institute (IRRI) developed new rice lines with adaptation to both anaerobic germination (AG) and submergence tolerance (Sub1) conditions. However, the characteristics of the water uptake pattern for these lines under anaerobic conditions are not yet sufficiently understood. Therefore, the objectives of this study were set to classify cluster group of germination ability and determine the role of genotypic of water uptake pattern during seed imbibition in the tolerance of rice seeds to anaerobic conditions by comparing the water uptake pattern of AG+Sub1 lines with other rice genotypes that were collected from different agro-ecological zones.

MATERIALS AND METHODS

Plant materials: This study was conducted in 2010 using fifty-eight contrasting rice (*Oryza sativa* L.) genotypes from the core collection of IRRI (Table 1). These genotypes were chosen

Table 1: List of rice genotypes used in the present study

Gen. No.	Genotype name	Country of origin	Gen. No.	Genotype name	Country of origin
1	ARC 10177	India	30	IR 29	Philippines
2	Baran Boro	Bangladesh	31	IR 42	Philippines
3	Bico Branco	Brazil	32	IR 48	Philippines
4	Black Gora (NCS12)	India	33	IR 56	Philippines
5	C 22	Philippines	34	IR 60	Philippines
6	Canela de Ferro	Brazil	35	IR 74	Philippines
7	CG 17	Senegal	36	ITA 212	Nigeria
8	Chanara 144	Pakistan	37	Jhona 26	Pakistan
9	Chianung Si-Pi 661020	Taiwan	38	Kasalath	India
10	DA 28	Bangladesh	39	Kataktara Da2	Bangladesh
11	Dholi Boro	Bangladesh	40	Khao Kap Xang	Thailand
12	Egyptian Jasmine	Egypt	41	LAC 23	Liberia
13	Fircoz	Iran	42	Mehr	Iran
14	FR13A	Philippines	43	Milyang 55	Korea
15	Gharib	Iran	44	Murungakayan 302	India
16	Giza 177	Egypt	45	N 22	India
17	Giza 181	Egypt	46	NP 125	India
18	Gotak Gatik	Indonesia	47	Pachehai Perumal	India
19	IR 06F148 (AG+Sub1)	Philippines	48	Padi Lebat	Indonesia
20	IR 06F168 (AG+Sub1)	Philippines	49	PTB 30	India
21	IR 06F393 (AG+Sub1)	Philippines	50	Rathal	Sri Lanka
22	IR 06F434 (AG+Sub1)	Philippines	51	Rikutou Nourin21	Japan
23	IR 06F459 (AG+Sub1)	Philippines	52	Sakha 103	Egypt
24	IR 06F463 (AG+Sub1)	Philippines	53	Shai-kuh	China
25	IR 06F561 (AG+Sub1)	Philippines	54	Surjamkuhi	India
26	IR 07F297 (AG+Sub1)	Philippines	55	Tadukan	Philippines
27	IR 07F323 (AG+Sub1)	Philippines	56	Tchampa	Iran
28	IR 22	Philippines	57	Trembese	India
29	IR 24	Philippines	58	WAB99-84 (FRF1)	Ivory Coast

based on a wide diversity of origins and their representation of a wide range of variability. Among them, nine lines (genotypic line numbers 19 to 27) were developed for anaerobic germination (AG) and submergence tolerance (Sub1) by the IRRI. In addition, FR13A (V14), with a designated Sub1 gene and IR42 (V31) without the Sub1 gene were used as model genotypes. The matured seeds of different genotypes were selected by pouring the seeds into sodium chloride solution with specific gravity 1.06 mg m^{-3}. Seeds that sunk in the solution were collected, washed 10 times with tap water and dried.

Assessment of water uptake by seeds: To determine the amount of water taken up by the seeds, two viable seeds from each genotype were marked and placed on a two layers of Whatman No. 1 filter papers in a petri dish of 9 cm diameter. The test was replicated 5 times with each genotype giving a total of 10 seeds for aerobic and anaerobic treatments. For the aerobic treatment, petri dishes were moistened with 8 mL of tap water to maintain moisture and arranged inside one big plastic container (85×56×20 cm). However, for anaerobic treatment, the petri dishes were placed inside the same size plastic container and the petri dishes were submerged at a 10 cm depth during the experiment. The containers were kept at 20°C and 80% of air humidity in dark conditions in a growth chamber. Before the start of the experiment, the germination capacity of all genotypes was confirmed at 28°C.

Sampling and calculation of water uptake: At 8, 16, 24, 32, 40, 48, 56, 64, 72, 80, 88, 96, 104, 112 and 120 h of imbibition, seeds were removed from the petri dishes and pressed carefully between paper towels to remove external surface water from the husk. Thereafter, each individual seed was weighed and placed in an oven at 80°C for 48 h to determine dry weight. To determine the initial moisture content of the seed, ten seeds from each genotype were weighed individually and dried at 80°C for 48 h. The water uptake was calculated as the difference between the seed wet and dry weights in reference to the dry weight multiplied by 100. The rate of water uptake was calculated by subtracting the water content of two effective consecutive measurements and dividing by the elapsed time. Because we expected variation in germination time among the tested genotypes, the germination time was calculated until 228 h. A seed was considered germinated when the emergent radicle reached 1 to 2 mm in length.

Statistical analysis: The data was statistically analyzed using a randomized complete block design with five replications. Analysis of variance (ANOVA) was used to analyze the data for the response of variables. When the differences were significant, Least Significant Differences (LSD) were calculated. Statistical analysis was done using the Costat system for Windows, version 6.311 (cohort software, Berkeley, CA, USA). The data on the water content in the seed at different times of seed imbibition was used for cluster analysis. Ward's minimum variance clustering method was used to classify genotypes into discrete clusters (Romersburg, 1988). The relationships between water uptake rate at different times of imbibition under aerobic and anaerobic conditions were drawn using regression equations calculated by Microsoft Excel 2003.

RESULTS

Based on the variation, the 58 genotypes were grouped into three clusters using Ward's method. The cluster technique clearly defined clusters on the basis of water content over different times of seed imbibition (from 0 until 120 h of seed imbibition) under aerobic (Fig. 1a) and anaerobic (Fig. 1b) conditions. The results of cluster analysis showed that clusters 1, 2 and 3

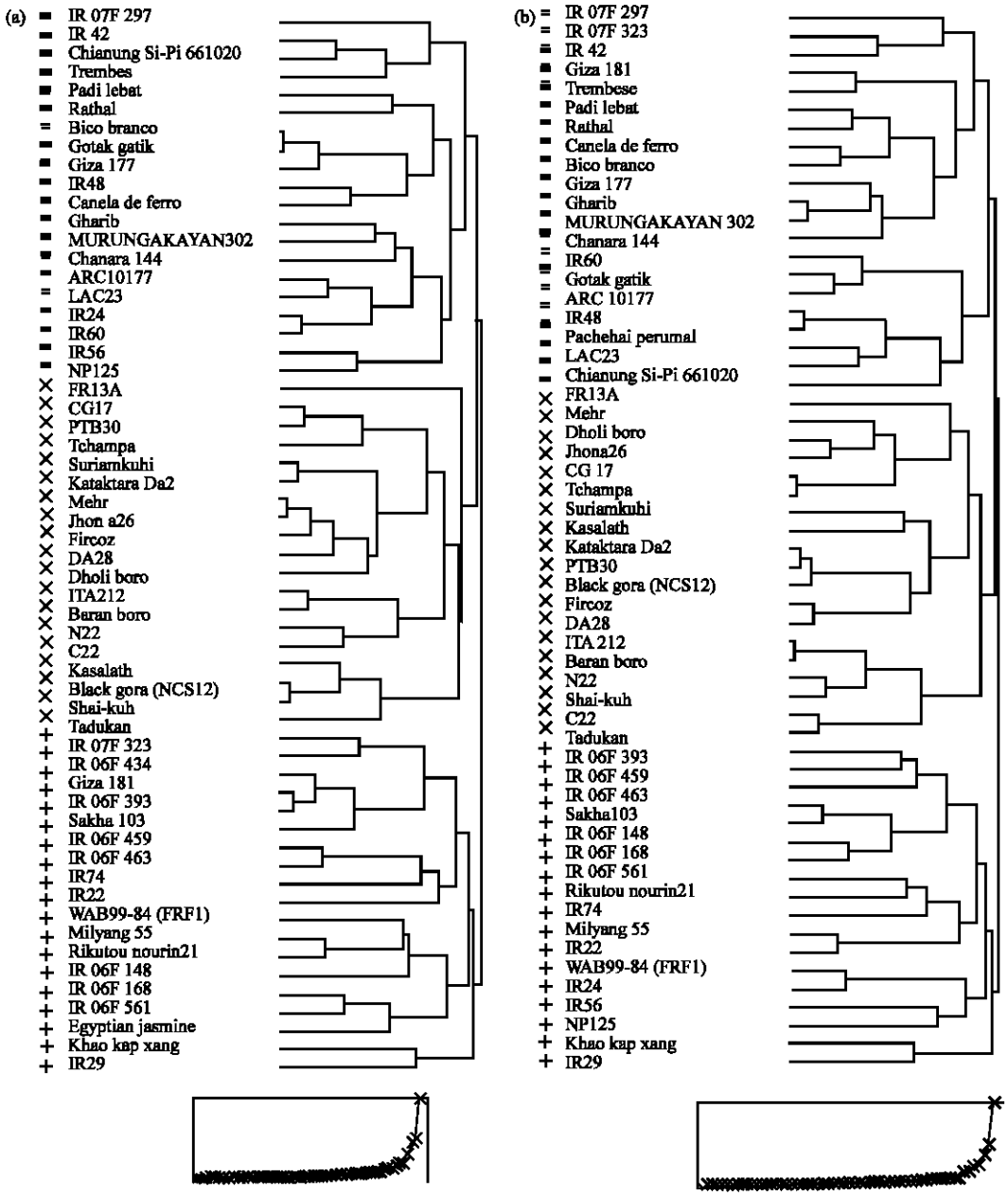

Fig. 1: Hierarchical cluster analysis of the 58 rice genotypes using water content in seeds over different times of seed imbibition (from 0 to 120 h of seed imbibition) under (a) aerobic and (b) anaerobic conditions

included 18, 19 and 21 genotypes under aerobic and 19, 19 and 20 genotypes under anaerobic conditions, respectively. It was interesting to note that most genotypes that mad up individual clusters under aerobic conditions also made the same individual clusters under anaerobic condition (Fig. 1a, b).

Figure 2 shows that the genotypes in cluster 3 which include most of the AG+Sub1 lines, attained a higher level of water content in the seeds at different times of seed imbibition than the genotypes in clusters 1 and 2. Most importantly, the genotypes in cluster 3 started absorbing water

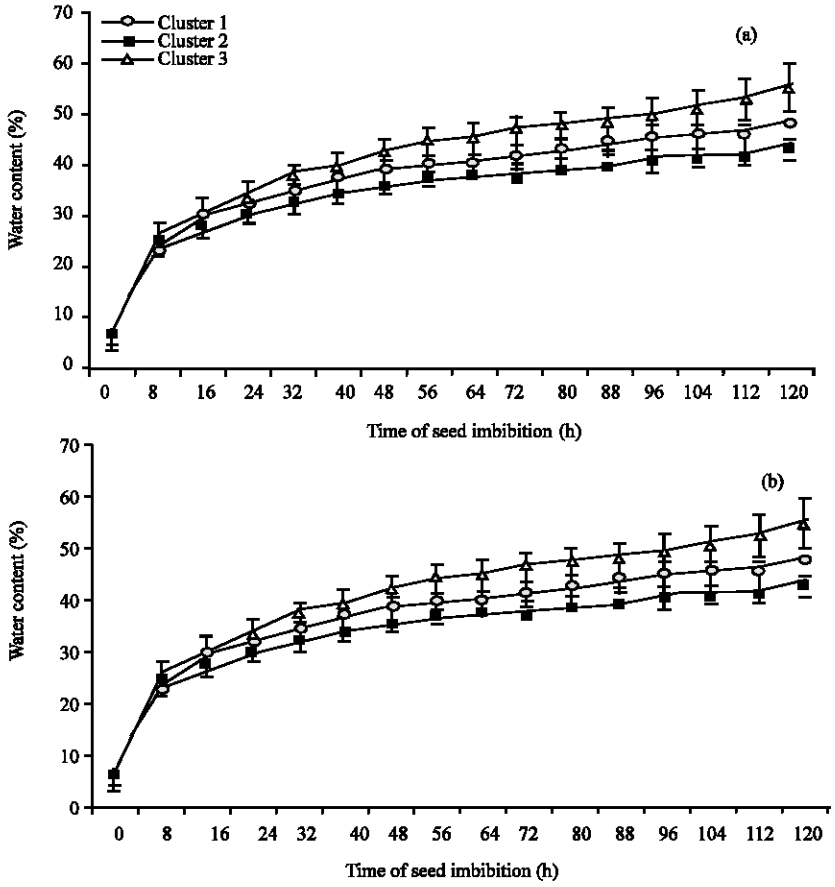

Fig. 2: Seed water content averaged over five replication±SE of the three clusters group of genotypes at different imbibition times under (a) aerobic and (b) anaerobic conditions. The value at each point was significantly different at $p<0.05$

very rapidly from the beginning under both conditions when compared with the genotypes in cluster 1 or cluster 2. For instance, the level of water content in the seeds of genotypes in cluster 3 were equivalent to 42.1% under aerobic condition and 41.2% under anaerobic condition within 48 h of imbibition, whereas the genotypes in clusters 1 and 2 reached this water content only after 80 and 120 h, respectively, under both conditions (Fig. 2a, b).

Cluster means values of the water uptake rate over different times of seed imbibition (0-48, 48-96 and 96-120 h of seed imbibition) and the 50% germination time of individual clusters under aerobic and anaerobic conditions are presented in Table 2. The genotypes thatformed cluster 3 were caracterized by rapid water uptake during the first 48 h of imbibition and rapid germination under both conditions. However, the water uptake rate during the first 48 h of imbibition of the genotypes in clusters 1 and 2 was decreased by 14.7 and 24.3% under aerobic conditions and 11.6 and 23.7% under anaerobic conditions, respectively, when compared with the genotypes in cluster 3. Again, water uptake rate between 96 and 120 h of seed imbibition also varied significantly among the three clusters under aerobic and anaerobic conditions. The water uptake rate between 96 and 120 h of seed imbibition of the genotypes in clusters 1 and 2 was decreased by 46.0 and 49.7% under aerobic condition and 23.9 and 54.8% under anaerobic condition,

Table 2: Water uptake rate over different times of seed imbibition (0-48, 48-96 and 96-120 h of seed imbibition) and 50% germination time for each individual clusters under aerobic and anaerobic conditions

	Clusters					
	Aerobic conditions			Anaerobic conditions		
Parameter	1	2	3	1	2	3
Water uptake rate (μg g^{-1} h^{-1})						
0-48 h	6.75±1.13	6.00±1.16	7.92±1.23	6.77±1.60	5.84±1.30	7.65±1.36
48-96 h	1.33±0.31	1.18±0.41	1.49±0.25	1.06±0.12	1.11±0.15	1.22±0.27
96-120 h	1.28±0.26	1.19±0.18	2.37±0.86	0.85±0.12	0.51±0.13	1.12±0.16
Germination time	121.1±10.4	124.6±12.9	108.0±12.4	149.2±15.2	164.8±15.1	136.2±18.1

Values are Mean±SE of individual time of water uptake rate and germination time

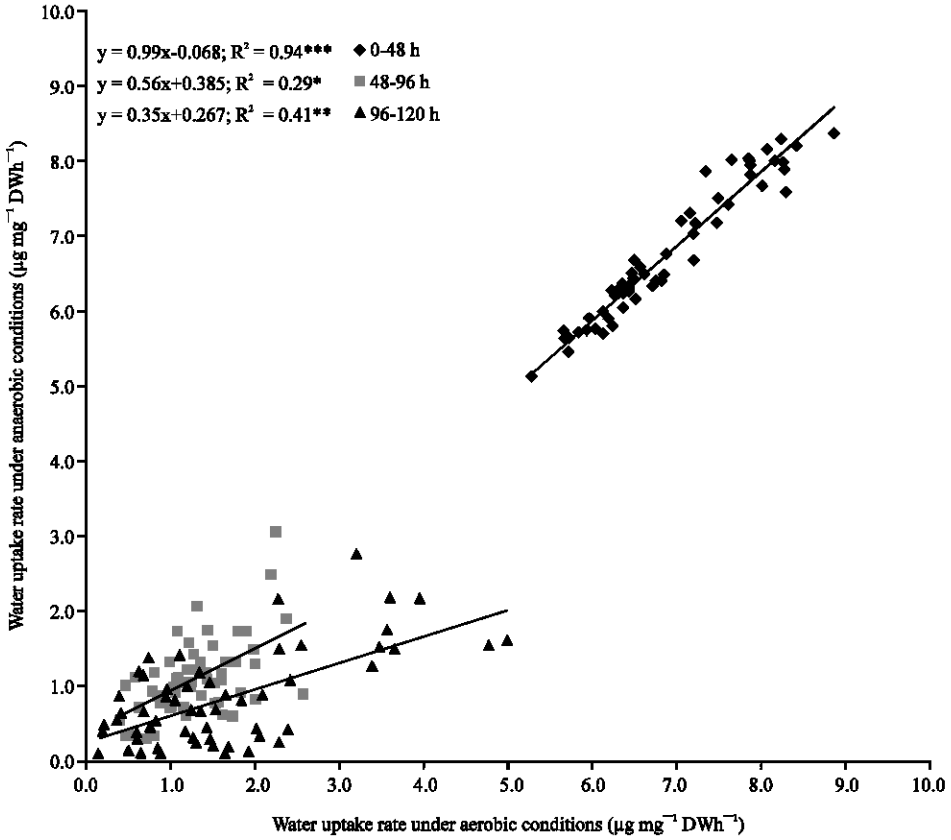

Fig. 3: Relationship between aerobic and anaerobic conditions for water uptake rate at different times of seed imbibition. Linear regression equation; *, ** and *** indicate significant differences at p = 0.05, 0.01 or 0.001, respectively

respectively as compared with the genotypes in cluster 3 (Table 2). Therefore, the germination time of genotypes in clusters 1 and 2 was increased by 10.8 and 8.7% for cluster 1 and 13.3 and 17.4% for cluster 2 under aerobic and anaerobic conditions, respectively, when compared with the genotypes in cluster 3.

Although, some genotypes were placed in cluster 3 which was characterized by rapid water uptake during the first 48 h of imbibition, the water uptake rate for these genotypes were found

to be higher and lower than those in the mean value for cluster 3 between 48 and 96 h and 96 and 120 h, respectively. For instance, the water uptake rate for Khao, Kap, Xang and Rikutou Nourin21 was increased by 32.4 and 34.1% under aerobic condition and 50.9 and 60.0% under anaerobic condition between 48 and 96 h and decreased by 58.9 and 38.8% under aerobic condition and 55.2 and 88.8 under anaerobic conditions between 96 and 120 h, respectively, when compared with the mean value of cluster 3 (data not shown).

The relationships between aerobic and anaerobic conditions were evaluated for water uptake rate at different times of imbibition (Fig. 3). Results of the regression analysis showed that a close relationship existed between aerobic and anaerobic conditions for each time of water uptake rate. However, these relationships were stronger for the water uptake rate between 0 and 48 h of imbibition than that between 48 and 96 h and 96 and 120 h of imbibition (Fig. 3).

Figure 4 reveals that, in general, the genotypes that germinated rapidly under aerobic condition also germinated rapidly under anaerobic conditions. In addition, the AG+Sub1 lines were germinated most rapidly under both conditions. The germination time for AG+Sub1 lines ranged from 72 to 88 h and from 72 to 128 h under aerobic and anaerobic conditions, respectively. The germination time for Egyptian Jasmine and Giza 181 genotypes were occasionally comparable to those for AG+Sub1 lines (Fig. 4a, b). However, germination time for some genotypes was significantly affected by anaerobic conditions. For example, the seed of ARC 10177, Chanara 144, Rikutou Nourin 21 and Tchampa were germinated at 156, 156, 180 and 180h under aerobic condition, while they germinated at 204, 228, 228 and 204 h under anaerobic condition, respectively (Fig. 4).

DISCUSSION

A useful depiction of the progress of germination has evolved around the time course of water uptake by a germinating seed (Nonogaki *et al*., 2010). Although water uptake during rice seed imbibition occurs generally with three phases such as rapid, plateau and post-germination phases, the time scale and duration of each phase varies considerably with respect to genotypes, type of stored carbon reserves and growth conditions (Kanmegne *et al*., 2010). Thus we hypothesized in this study that significant genotypic differences in regulation of water uptake rate during each phase may play an important role in the ability of rice seeds to germinate faster under anaerobic conditions. In this study, we chose the intervals between 0 and 48, 48 and 96 and, 96 and 120 h of seed imbibition to represent the pattern of water uptake during seed germination under aerobic and anaerobic conditions.

This study indicated that most AG+Sub1 lines were placed in cluster 3 which was characterized by rapid water uptake during the first 48 h of imbibition under aerobic and anaerobic conditions (Fig 1a, b and 2). Furthermore, the strong negative correlation between water uptake rate between 0 and 48 h of seed imbibition and germination time ($r = -0.46$, $p<0.001$) also confirmed that rapid water uptake during the first 48 h of imbibition probably plays a key role in the ability of AG+Sub1 lines to germinate faster under anaerobic conditions. These findings were in good agreement to those reported by Olisa *et al*. (2010) who found that rapid imbibition was a cause of reduced germination time and scarification is not necessary for pigeon pea species as it accelerated imbibition. This could be explained by the fact that rapid water uptake during the first 48 h may lead to a rapid activation of α-amylase which plays an important role in the degradation of starch into soluble sugars as the main substrate necessary for generating the energy required for growth

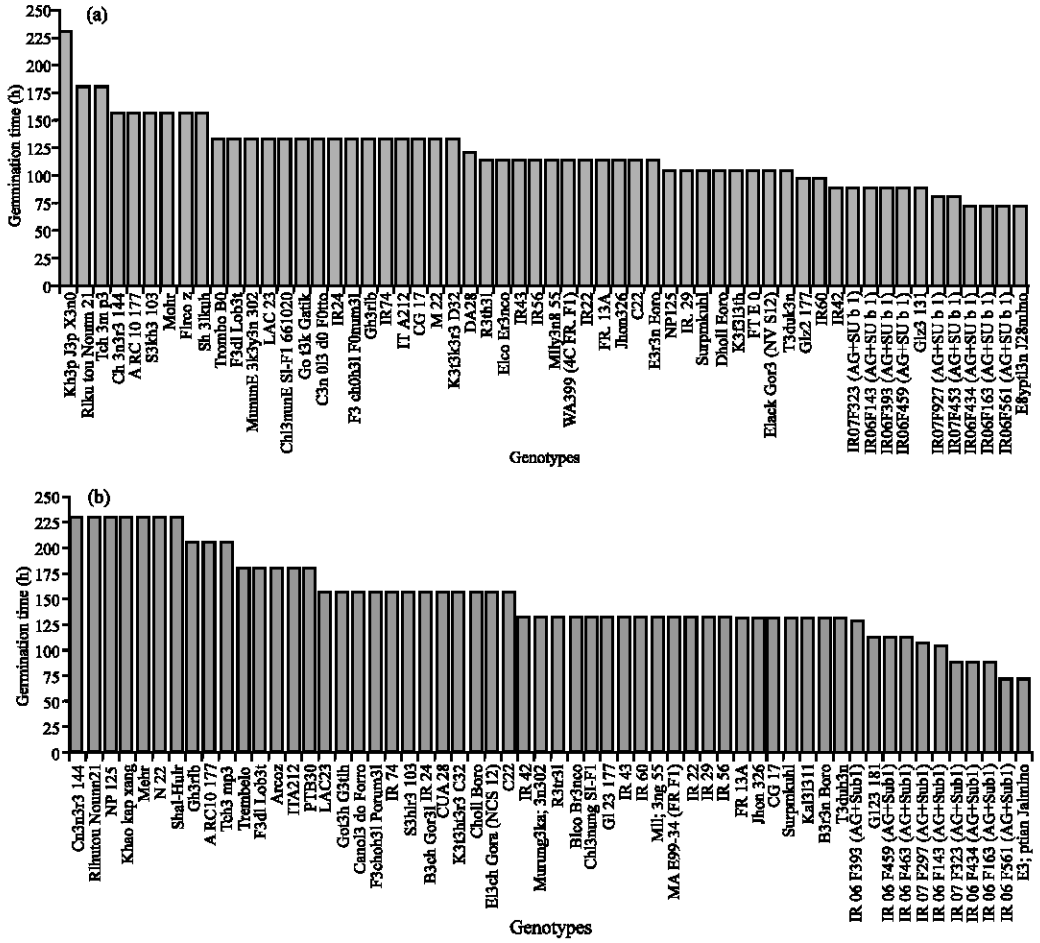

Fig. 4: Germination time of 58 genotypes under (a) aerobic and (b) anaerobic conditions

and maintenance processes. In addition, rapid water uptake under anaerobic conditions may lead to rapid sugar mobilization between the endosperm and embryo. Indeed, Ismail *et al.* (2009) reported that the ability of tolerant genotypes to degrade starch into soluble sugars under anaerobic conditions probably plays a key role in their ability to grow faster under these conditions. Huang *et al.* (2003) reported that when seeds were imbibed and grown in anoxia, IR22 (anoxia-intolerant) grew much slower and had lower soluble sugar concentrations in seeds than Amaroo (anoxia-intolerant). These results implied that faster water uptake during the first 48 h of imbibition may play a key role in the ability of tolerant rice genotypes to germinate rapidly even under anaerobic conditions.

Although some of the tested genotypes exhibited a higher water uptake rate during the first 48 h of imbibition, they germinated slowly under anaerobic conditions. This is probably because these genotypes showed a continuous increase in water uptake rate between 48 and 96 h followed by decrease between 96 and 120 h like genotypes: Khao, Kap, Xang and Rikutou Nourin21 which germinated after 228 h of imbibition under anaerobic conditions, or showed a lower water uptake rate after 48 h of imbibition such as genotypes clusters 1 and 2 (Table 2). However, most AG+Sub1 lines which germinated rapidly under both conditions, exhibited a lower and higher rate of water uptake again between 48 and 96 h, and 96 and 120 h of imbibition, respectively. The

results also showed a strong positive ($r = +0.36$, $p<0.01$) and negative ($r = -0.61$, $p<0.001$) correlation between germination time and water uptake rate between 48 and 96 h and between 96 and 120 h of imbibition, respectively, under anaerobic condition. The decrease in water uptake rate for AG+Sub1 lines between 48 and 96 h implied that the hydrolysates of endosperm reserves are translocated to the embryo and new cell materials are re-synthesized there under anaerobic conditions. Therefore, the increase in water uptake rate of AG+Sub1 lines between 96 and 120 h of imbibition is closely related with active growth of the plumule and radicle after germination. The seeds of AG+Sub1 lines were germinated at, on average, 80.9 and 100 h under aerobic and anaerobic conditions, respectively (Fig. 4). However, a continuous decrease in water uptake rate especially between 96 and 120 h of imbibition, as seen for the genotypes in clusters 1 and 2 (Table 2), may lead to slow rates of sugar mobilization in the endosperm under anaerobic conditions. Therefore, the germination time of clusters 1 and 2 was increased by 10.8 and 8.7% for cluster 1 and 13.3 and 17.4% for cluster 2 under aerobic and anaerobic conditions, respectively, when compared with the genotypes in cluster 3 (Table 2). In addition, a continuous increase in water uptake rate after 48 h of imbibition like Khao, Kap, Xang and Rikutou Nourin21 genotypes may lead to injury of cells in the aleurone layer in the endosperms of these genotypes which in turn decreased the synthesis of hydrolytic enzymes. Huang *et al.* (2003) reported that this lesion was healed also under aerobic conditions. Therefore, results of this study showed that both genotypes were germinated after 180 and 228 h of imbibition under aerobic conditions and 228 h of imbibition under anaerobic conditions, respectively (Fig. 4), although they take up water rapidly during the first 48 h like AG+Sub1 lines. These results confirmed that the regulation of water uptake during the rice seed imbibition may play an important role in the tolerance mechanisms of AG+Sub1 lines for anaerobic conditions during germination.

Results in this study showed that the model genotype (FR13A), with a designated Sub1A gene and genotype (IR42) without the Sub1A gene, were placed in cluster 1 and 2 which were both characterized by slower germination than cluster 3 under anaerobic conditions (Fig. 1b, 4). This indicated that the characteristics of water uptake for FR13A were much closer to those of IR42. The findings obtained in this study were in good agreement with those reported by Magneschi and Perata (2009), who reported that rice's capacity to germinate under complete anoxia cannot be explained in terms of Sub1 genes. Fukao *et al.* (2006) and Xu *et al.* (2006) also found that M202 and Nipponbare genotypes, neither of which has the Sub1 gene, display a vigorous germination under anoxia. This implies that the Sub1 locus in rice is not relevant for tolerance to anaerobic germination.

CONCLUSION

In conclusion, the present results verified that the regulation of water uptake during rice seed imbibition could be involved in the ability of rice seeds to germinate faster under anaerobic conditions. The seed of most of AG+Sub1 which were developed for Anaerobic Germination (AG) and submergence tolerance (Sub1) conditions, started absorbing water more rapidly from the beginning under aerobic and anaerobic conditions, therefore, these genotypes were characterized by rapid germination under both conditions.

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