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**A simple, rapid and reliable bioassay for evaluating seedling vigor under
submergence in *indica* and *japonica* rice (*Oryza sativa* L.)**

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Summary

Submergence is a major stress causing yield losses particularly in the direct-seeded rice cultivation system and necessitates the development of a simple, rapid and reliable bioassay for a large scale screening of rice germplasms with tolerance against submergence stress. We developed two new bioassay methods that were based primarily on the seedling vigor evaluated by the ability of fast shoot elongation under submerged conditions, and compared their effectiveness with two other available methods. All four bioassay methods using cultivars of 7 *indica* and 6 *japonica* types revealed significant and consistent cultivar differences in seedling vigor under submergence and/or submergence tolerance. *Japonica* cultivars were more vigorous than *indica* cultivars, with Nipponbare being the most vigorous. The simplest test tube method showed the highest correlations to all other methods. Our results suggest that seedling vigor serves as a submergence avoidance mechanism and confers tolerance on rice seedlings to flooding during early crop establishment. A possible relationship is discussed between seedling vigor based on fast shoot elongation and submergence tolerance defined by recovery from submergence stress.

Key words: *indica*, *japonica*, rice (*Oryza sativa* L.), seedling vigor, submergence tolerance

Introduction

Rice cultivation system has ever been undergoing various changes in response to economical and technological factors in farming. A recent change occurring in Asian countries is a shift from the seedling-transplantation system to the direct-seeding system for rice establishment. This is due primarily to the rising cost of labor that has provided the direct seeding system with economic incentives. The fact that large tracts of rice fields in rain-fed lowland areas are frequently flooded during early seedling establishment makes the development of sufficient tolerance against flooding a particularly important and urgent breeding target (Dingkuhn *et al.* 1992).

Seedling vigor is defined as the ability of seedlings to emerge rapidly from soil or water (Heydecker 1960). Seedling vigor is important as the sum of all attributes supporting the early seedling growth particularly under unfavorable field conditions. In the areas where rice seeds are sown directly in the fields prone to flood, seedling vigor is

a determinant of the optimum crop establishment (Redona and MacKill 1996). High germination rate and fast shoot and root elongation are the major traits that are closely related to seedling vigor (Williams and Peterson 1973; Sasahara *et al.* 1986). Deepwater or floating rice in fact exhibits extreme elongation capacity under submergence (Kende *et al.* 1998), and hence it can escape complete submergence or full inundation by rapid growth under water. Seedling vigor, which likely plays a key role in avoiding or escaping flooding stress, is also an important factor in the drill-seeded planting system in rice (Dilday *et al.* 1990). When shoot emergence delays due to low seedling vigor, the rate of seedling mortality increases (Peterson *et al.* 1978).

In contrast to seedling vigor, submergence tolerance and/or anaerobic tolerance generally refers to the tolerance against temporal flash-flooded conditions, when the water level rises quickly and covers the whole plants for a period of not more than 2 weeks and then water recedes afterwards. Submergence tolerance is characterized by the ability of plants to recover after flash flooding at various stages of growth (Kamolsukyunyong *et al.* 2001). The recovery involves recuperation of plants damaged by sudden flooding. The bases for the submergence tolerance have been well established as being associated with high initial carbohydrate contents, high levels of chlorophyll retention, and minimum growth under submerged conditions (Ito *et al.* 1999;

Ram *et al.* 2002; Jackson and Ram 2003; Das *et al.* 2005; Takeshi *et al.* 2006).

Both seedling vigor and submergence tolerance are difficult traits to measure under field conditions because of the involvement of many uncontrollable environmental factors. Setimela *et al.* (2005) pointed out such difficulty in separating heat stress and water stress on seedling vigor (optimum potential of coleoptile growth) in the field. Redona and Mackill (1996) showed that a screening method based on seedling vigor was effective for drill seeding under field conditions. Nevertheless, efforts have been directed for developing effective and useful greenhouse and laboratory screening methods (Redona and Mackill 1996). The slant-board test developed by Jones and Peterson (1976) was first used in studies of seedling vigor under submergence in relation to semi-dwarfism in rice (McKenzie *et al.* 1980). This test was applied in screening for rice breeding lines in California (McKenzie *et al.* 1994). Xu and Mackill (1996) developed a seedling recovery method for evaluating submergence tolerance under full inundation due to flash floods in rice, while Yamauchi and Winn (1996) developed a seedling emergence method for screening of fast seedling emergence under flooding.

A large reservoir of rice genetic resources are available including both *indica* and *japonica* types, from which promising germplasms with desirable traits can be selected. Our present study aimed at developing a simple, rapid and reliable bioassay method for

screening rice germplasms with high seedling vigor or high ability of fast shoot elongation under submerged conditions using seedlings at the stage of germinating embryo-through-seedling development. We obtained results supporting that seedling vigor plays a key role in the submergence avoidance mechanism.

Materials and methods

Plant materials

Thirteen rice (*Oryza sativa* L.) cultivars consisting of 7 *indica* and 6 *japonica* types were used (Table 1). A pilot experiment using the seedling recovery method of Xu and Mackill (1996) and a number of *indica* cultivars showed that an Indian local cultivar ASD1 was the most vigorous and tolerant to submergence stress and thus it was used as an *indica* tolerant check in the present study. In addition, 3 *indica* cultivars (tolerant FR13A, susceptible IR42 and Kasalath) were used as known check cultivars. De-hulled seeds were subjected to overnight imbibition under running water to allow synchronous germination. Imbibed seeds were surface sterilized with 1 % (w/v) solution of sodium hypochlorite for 10 min and rinsed in distilled water as described by Boamfa *et al.* (2003).

Sterilized seeds were allowed to germinate in wet petri dishes placed in an incubator at 28 °C in the dark, and they were washed everyday with distilled water for further stimulation of uniform germination. Germinating seeds at the pigeon breast stage (pre-germinated seeds of about 3-day-old) were selected and used in the bioassays.

Bioassay methods

In the following 4 bioassay methods, 10 pre-germinated seeds were used per cultivar. All experiments were laid out according to a completely randomized design with three replications.

Test tube method. Pre-germinated seeds were placed in a glass test tube (2.5 cm in diameter and 15 cm in height) filled with 10 cm deep distilled water, and incubated at 28 °C in the dark without changing water. At the 5th day of incubation, shoot (coleoptile) lengths were measured (Fig. 1A).

Water-lodged soil method. Pre-germinated seeds were sown in paddled soil filled in cells (4 x 5 cm) in a plastic tray (27.5 x 27.5 x 4.5 cm) at the depth of 3 cm from the soil surface. Each cell represented an experimental unit. The tray was then immersed inside a glass container (31 x 42 x 20.5 cm) filled with 3 cm deep water above the soil surface (Fig. 1B) and placed in a greenhouse. To obtain comparable data with those

from the test tube method, seedlings were removed from the soil at the 5th day and their shoot lengths were measured.

Seedling recovery method. This is a modified version of the original method of Xu and Mackill (1996) that was developed for evaluating submergence tolerance. Pre-germinated seeds were sown in soil filled in a metal tray (27.5 x 27.5 x 2.5 cm) at the depth of 1 cm and grown until shoots reached about 10 cm in length (about 8 days after sowing). The seedlings were submerged in 40 cm deep water (from the soil surface to the water surface) in a plastic bucket (31 x 34 x 43 cm) for another 10 days in a biotron at 25 °C with a 16-h photoperiod (16hL/8hD) at a light intensity of 110-120 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ provided by cool white fluorescent lamps (standard condition). The plastic bucket was partially shaded with a thin white cloth to reduce the light intensity. Submergence tolerance was individually scored after a one-week recovery period in a greenhouse using the modified version of the scale adopted by Xu and Mackill (1996) as shown in Figure 2.

Seedling emergence method. This method is basically according to Yamauchi and Winn (1996) that was devised for evaluating seedling vigor index. Vigor Index was calculated based on the number of seedlings emerged from the water surface after the fixed time points, i.e. at the 2nd, 4th and 6th days after sowing. Seedling emergence and subsequent

crop establishment is suppressed by substances like ferrous irons produced and accumulated in flooded paddy soils (Hagiwara *et al.* 1993). To provide reductive soils in this bioassay, we kept soils under submerged conditions for 15 days prior to the test. Paddled soil filled in cells (4 x 5 cm) in plastic tray (27.5 x 27.5 x 4.5 cm) was submerged for 15 days prior to seeding in a glass container (31 x 42 x 20.5 cm). Pre-germinated seeds were then sown in the reduced soil at the depth of 3 cm and the tray containing the pre-germinated seeds was submerged in 5 cm deep water above the soil surface, and kept in a biotron under the standard condition. The following two parameters were used in this bioassay. Percentages of seedling emergence were taken at the 2nd, 4th and 6th days of submergence. Seedling vigor index was according to a formula of Yamauchi and Winn (1996).

Statistical analysis. Analysis of Variance (ANOVA) was performed using the Microsoft Excel Program. Least significance differences (LSD) were calculated using the software program, Analyze-it + General 1.68 version. Correlations among the parameters were calculated according to Gomez and Gomez (1984).

Results

Variations in seedling vigor under submergence. Highly significant cultivar differences were found in all of the five parameters evaluated by four bioassay methods (Table 1). Comparisons were made between the vigorous *indica* check ASD1 and each of *indica* and *japonica* cultivars by LSD test. The test tube method (Fig. 1A) and the water-lodged soil method (Fig. 1B), in both of which the shoot length at the 5th day of submergence was used as a common parameter, gave significant differences among all the cultivars either at the 1% or 5 % significance level. The length of the shoot of *japonica* cultivars (5.44 cm) was not significantly different from that of the *indica* cultivars (4.71 cm). The water-lodged soil method was more severe than the test tube method, thus the average shoot lengths of both *japonica* (4.23 cm) and *indica* (3.76 cm) were shorter than those obtained by the test tube method. The difference in the mean shoot length of the *indica* and *japonica* cultivars was not statistically significant. In both methods, Nipponbare and Kamenoo were the most vigorous and their seedling vigor was comparable with tolerant check ASD1, while susceptible check PSBRc102 was most susceptible.

In the seedling recovery method, arbitrary scorings were made after 7 days of recovery period (Fig. 2). Nipponbare, Kamenoo and ASD1 showed the score of 2

(tolerant), while that of PSBRc102 was 9 (susceptible). An average score of the *indica* type (5.3) was significantly higher (more susceptible) than that of the *japonica* type (2.7) at the 1% level.

In the seedling emergence method, we first sowed pre-germinated seeds in 5 cm deep soil and flooded with 5 cm deep water from the soil surface. Percentage values of shoot emergence from the water surface were recorded at the 4th day after sowing. Under this condition, Nipponbare showed 40-70 % seedling emergence, whereas no emergence occurred in all other cultivars including ASD1. To reduce the severity of the treatment, pre-germinated seeds were sown in 3 cm deep soil and flooded with 5 cm deep water. In this second test, the *indica* type showed a mean emergence of 50.9 %, while that of *japonica* was 61.7 %. The difference between the *indica* and *japonica* cultivars was significant at the 1% level. Nipponbare was the most vigorous showing 100 % seedling emergence, while PSBRc102 was the most susceptible showing 20 % seedling emergence.

The mean vigor index of *indica* was 2.85 (more susceptible), significantly different ($P<0.01$) from that of *japonica* (4.13). Nipponbare and ASD1 were the most vigorous and PSBRc102 the most susceptible according to this parameter.

Correlations among the bioassay methods. Correlation analysis was conducted among all possible pairs of the five parameters. The analysis revealed R-values ranging from 0.51 to 0.95 (Tab. 2). The test tube method showed significant correlations with all other parameters except for percent seedling emergence. The water-lodged soil method also showed significant correlations with all other parameters except for percent seedling emergence. Vigor index showed significant correlations with all other methods. On the other hand, percent seedling emergence showed a significant correlation only with vigor index. Based on these results, the test tube method was considered to be the most simple, rapid and reliable bioassay for screening seedling vigor of rice under tested submergence conditions.

Validation of the test tube method using submergence tolerant and susceptible checks.

A significant correlation between the test tube method and the seedling recovery method implied an association between seedling vigor under submergence and submergence tolerance. To examine this, Nipponbare and PSBRc80 were used for bioassay along with a standard submergence tolerant cultivar FR13A and a susceptible cultivar IR42. An Indian *indica* cultivar Kasalath was also included in the bioassay. The test tube method and the method of Xu *et al.* (2006) that was a modified version of Xu and

Mackill (1996) devised for submergence tolerance were applied to the five selected cultivars. In the test tube method, Nipponbare showed the longest shoot followed by the submergence tolerant check FR13A (Fig. 3). Kasalath ranked third followed by PSBRc80, and the submergence susceptible check IR42 was indeed the most susceptible showing the slowest shoot elongation rate. In the method of Xu *et al.* (2006), in which 14-days-old seedlings were submerged for 14 days and de-submerged for another 14 days before the recovery rate was measured, 33.3 % of Nipponbare seedlings and 13.3 % of FR13A seedlings showed recovery (Fig. 4). Ten percent of PSBRc80 seedlings recovered but no recovery was observed in Kasalath and IR42. Nipponbare and FR13A were the most submergence tolerant. On the other hand, Kasalath and IR42 were completely dead in this bioassay, showing their high susceptibility.

Discussion

Seedling vigor of *indica* and *japonica* rice cultivars under submergence was evaluated by two newly devised bioassay methods, i.e. test tube and water-lodged soil methods, both of which were based primarily on the ability of fast shoot elongation. In the bioassay

we used pre-germinated seedlings because we tried to evaluate shoot elongation ability rather than germination ability under submerged conditions. Our comparative study of the effectiveness of these methods together with two other available methods, which were previously developed for evaluating submergence tolerance (Xu and Mackill 1996) and for fast seedling emergence (Yamauchi and Winn 1996), revealed significant and consistent variations in both *indica* and *japonica* cultivars. The test tube method showed high correlations with all other methods (Table 1). Based on three among five parameters the *japonica* cultivars were shown to be more vigorous than the *indica* cultivars. A standard *japonica* cultivar Nipponbare was the most vigorous, while an *indica* cultivar PSBRc102 was the weakest. Based on these results, the test tube method was considered to be the most simple, rapid and convenient bioassay that can be used in mass screening for seedling vigor under submergence. This simple method should also be useful in mapping quantitative trait loci controlling seedling vigor under submergence by enabling rapid and accurate phenotyping of the mapping population.

Increases in plant height was used as an indicator of flooding tolerance in *Brassica rapa* L. (Daugherty and Musgrave 1994). Won and Yoshida (2000) used fast shoot elongation growth as a basis for screening rice cultivars at low dissolved oxygen levels and suggested that faster shoot elongation was an adaptive response of water-seeded rice

for acquiring oxygen. Vriezen *et al.* (2003) also suggested that maintenance of fast growth under water is a trait indispensable for survival under prolonged submergence. Seedling vigor is related to early germination, and good emergence and stand establishment in sorghum and other field crops (Cisse and Ejeta 2003). Our results clearly suggest that the ability of fast shoot elongation is a key factor in determining seedling vigor under submergence in both *indica* and *japonica* rice. It is thus reasonable to assume that fast shoot elongation enables rice seedlings to rapidly reach the water surface and allows taking up oxygen into shoot tissues. In this context, fast shoot elongation can be considered as the major submergence avoidance or escape mechanism. This trait is important for seedling survival when shallow floods occur after direct seeding or transplantation, or in deepwater areas where deep floods stagnates for longer durations and thus fast growth becomes an advantage to keep pace with rising water.

By contrast, Setter and Laureles (1996) reported that reduced elongation growth conferred a beneficial effect on submergence tolerance in rice. They showed that a gibberellin-deficient mutant of rice, which had a little elongation ability during submergence, was more tolerant than the wild type when plants were submerged at the time of equal initial dry weights and carbohydrate levels. Setter *et al.* (1994) studied relationship between the coleoptile elongation and alcoholic fermentation in germinating

seeds of *indica* rice subjected to submergence stress, and showed that when elongation growth underwater was limited by application of a gibberellin biosynthesis inhibitor, paclobutrazol, the percentage rate of seedling survival increased by as much as 50 times in most of the cultivars they studied. Accordingly they hypothesized that fast elongation growth requires a high level of energy at the expenses of the maintenance of an appropriate energy level required for cell and tissue integrity and hence it reduces survival under submergence stress. They further discussed the possibility that a slow rate of fermentation is beneficial because of the resulting slow rate of consumption of reserved carbohydrates under a long period of submergence. No rice cultivars could survive when they were submerged for more than 15 days, because alcohol fermentation could not supply enough energy and photosynthetic carbon assimilation was largely prevented by such a prolonged period of submergence (Boamfa *et al.* 2003).

It has been suggested that seedling vigor under submergence defined by rapid shoot elongation and submergence tolerance by high recovering ability involves different mechanisms, although they are not necessarily mutually exclusive. Xu *et al.* (2006) reported that submergence tolerance was controlled by the particular genotype of the complex locus *Sub1*, with ectopic expression of *Sub1A* derived from a tolerant cultivar FR13A being sufficient to enhance tolerance. Nipponbare was concluded to be

intolerant to submergence stress based on the absence of the *Sub1A* locus in its genome. They also showed that *Adh1* transcript levels increased more in the tolerant line FR13A than in Nipponbare. A different result, however, was reported by Kato-Noguchi (2001), in which Nipponbare was more tolerant showing higher *Adh* activity in coleoptiles of germinating embryos submerged for 48 hours than intolerant cultivars. To find an answer to the question if seedling vigor under submergence is related to the submergence tolerance under flash-flooded conditions, we compared the test tube method and the method of Xu *et al.* (2006). We observed a good agreement between the levels of seedling vigor (Fig. 3) and submergence tolerance (Fig. 4) among the tested materials including known submergence tolerant and susceptible *indica* lines and a *japonica* cultivar Nipponbare. Nipponbare was shown to be the most vigorous in both methods. The tolerance of Nipponbare was repeatedly confirmed in our experiments using Nipponbare obtained from different sources. Although this conflicting result might be ascribed to some unknown experimental conditions, our result suggests a possible association between seedling vigor evaluated based on fast shoot elongation and submergence tolerance evaluated by slow sustained metabolism and high recovery from submergence stress. This point must be further clarified by more rigorous and comprehensive study using a larger set of germplasm with varying levels of seedling

vigor and submergence tolerance.

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Figure legends

Figure 1. Two bioassay methods devised in this study. (A) Test tube method. From left to right: ASD1 (*indica* tolerant check), PSB Rc62 and PSB Rc102 (*indica* susceptible checks). (B) An over-view of water-lodged soil method. Inlet shows a side-view of plastic cells.

Figure 2. Seedling recovery method modified after Xu and Mackill (1996). The arbitrary scoring system (1-9) was based on the degree of recovery.

Figure 3. Test tube method evaluating seedling vigor using known submergence tolerant (FR13A) and susceptible (IR42) check cultivars.

Figure 4. Seedling recovery method by Xu et al. (2006) applied for evaluating submergence tolerance to known submergence tolerant (FR13A) and susceptible (IR42) check cultivars.

Table 1. Seedling performance of 13 rice cultivars evaluated by five parameters in four bioassay methods. Shoot lengths were taken after 5 days while scoring was done after one week recovery. Percent seedling emergence was taken on the 6th day, and the vigor index (VI) was calculated based on the sum of 2nd, 4th and 6th day after Yamauchi and Winn (1996).

Cultivar	Mean Shoot Length (cm)		Seedling Recovery	Seedling Emergence	
	Test Tube	Water-lodged Soil	Score (1-9)	% Seedling Emergence	Vigor Index
PSB Rc46	4.82*	3.84**	5**	43.3**	1.92**
PSB Rc54	5.34*	4.5*	3*	50**	2.53**
PSB Rc62	4**	2.98**	7**	40**	1.94**
PSB Rc80	4.58**	3.5**	6**	80 ^{ns}	3.78**
PSB Rc28	4.79*	3.77**	5**	43.3**	2.36**
PSB Rc102	2.9**	2.19**	9**	20**	0.83**
ASD 1	6.58	5.57	2	80	6.61

Mean of <i>indica</i>	4.71	3.76	5.3	50.9	2.85
Nipponbare	6.82 ^{ns}	5.85 ^{ns}	2 ^{ns}	100**	7 ^{ns}
Kamenoo	6.73 ^{ns}	5.73 ^{ns}	2 ^{ns}	80 ^{ns}	5.35**
Nakachaku	4.54**	3.41**	5**	76.67**	4.96**
Noujouho	5.43*	4.43**	3**	43.3**	2.93**
Fusakichi	4.25**	3.26**	5**	23.33**	1.43**
Waseshinriki	4.88*	3.89**	5**	46.67**	3.13**

Mean of <i>japonica</i>	5.44	4.23	2.7	61.7	4.13
cv(%)	9.42	11.48	8.31	7.92	7.51
LSD _{5%}	0.80	0.78	0.64	7.43	0.43
LSD _{1%}	1.87	1.06	0.87	10.04	0.59
F-test	**	**	**	**	**

*/** - significantly different from the tolerant standard ASD 1 at the 1% and 5% levels, respectively

^{ns} - not significantly different from ASD1

Table 2. Correlation among five parameters obtained by four bioassay methods using *indica* and *japonica* rice cultivars.

Bioassay	Water-lodged	Seedling	% Seedling	Vigor
method	Soil	Recovery	Emergency	Index
Test tube	0.9518**	0.9183**	0.5474 ^{ns}	0.6431**
Water-lodged Soil		0.9437**	0.5112 ^{ns}	0.6149*
Seedling Recovery			0.4477 ^{ns}	0.5787*
% Seedling Emergency				0.8811**

*/** - significant at the 1% and 5% levels, respectively

^{ns} - not significant

A



B



Figure 1. Manangkil et al.



Figure 2. Manangkil et al.

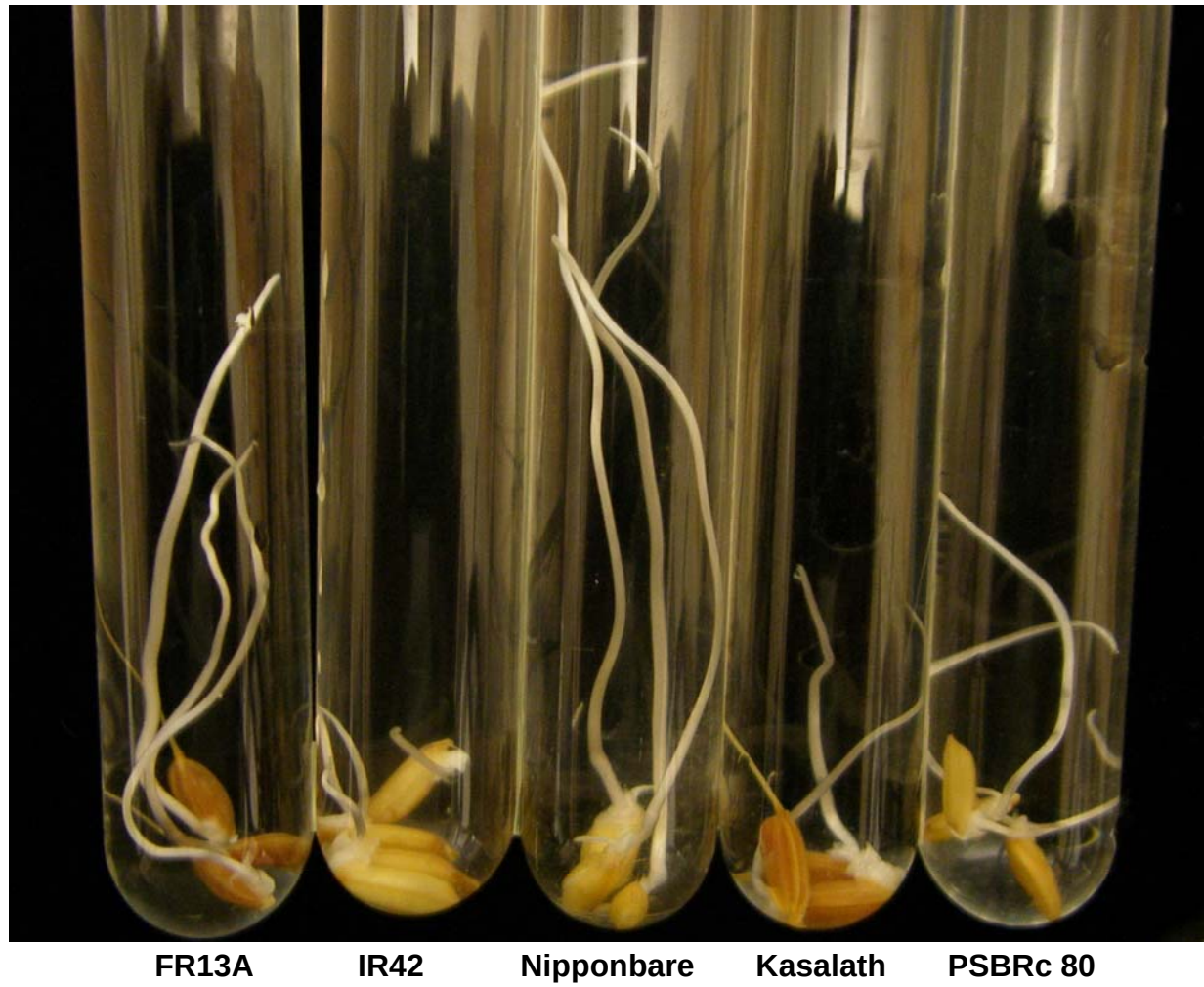
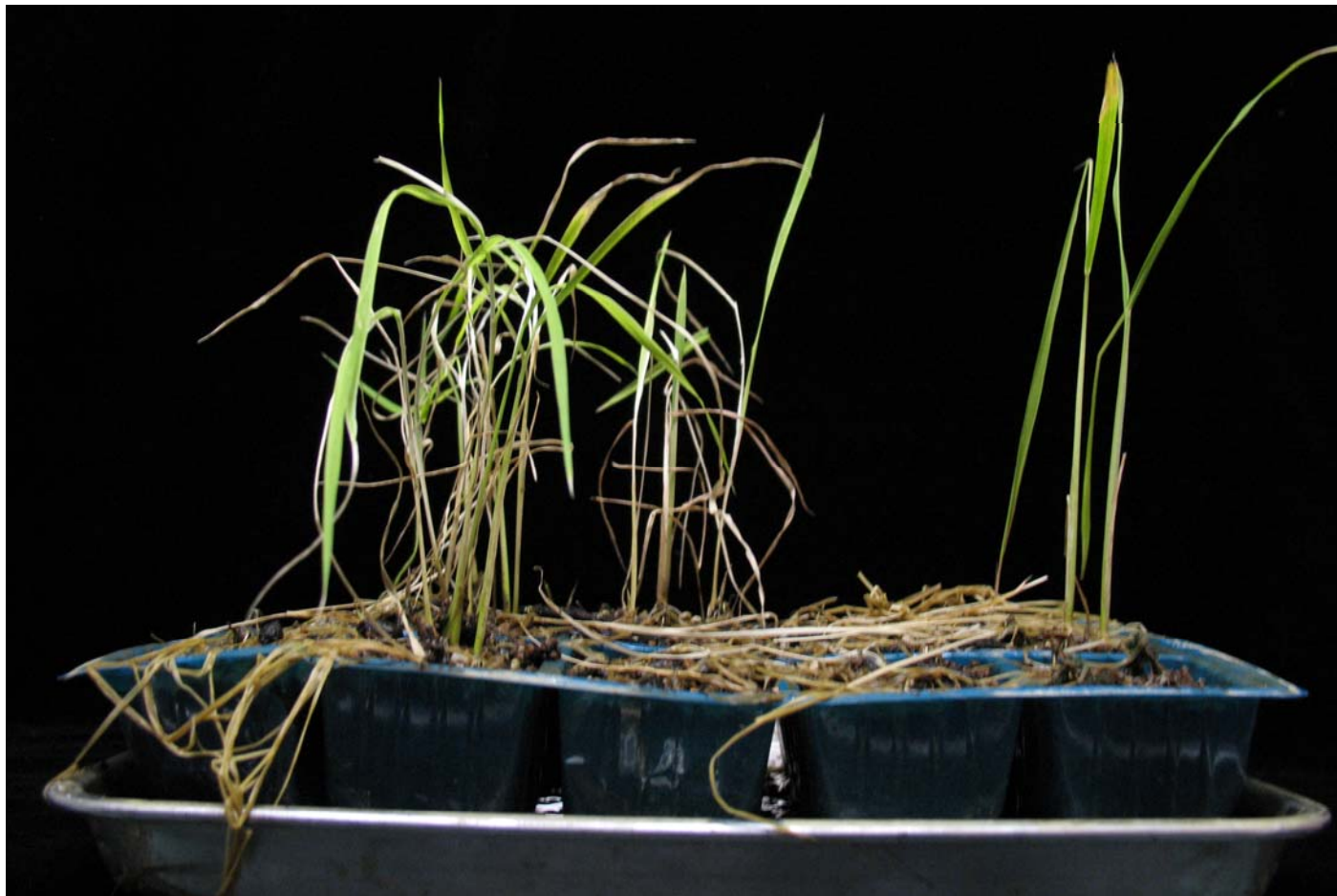


Figure 3 Manangkil et al.



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Figure 4. Manangkil et al.