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A COMPARATIVE STUDY ON FLOOD TOLERANCE OF SHORT AND TALL STATURE RICE CULTIVARS WITH *Sub1* DURING SEED GERMINATION AND SEEDLING STAGE

BHARATHKUMAR S.*, JITENDRA K., PRAGNYA P.J., GAYATRI G., ARCHANA B., SAUMYA R.B., MITADRU M., RAVINDRA D., BARADA P.P., SHAKTI P.M. AND REDDY J.N.

Crop Improvement Division, ICAR-Central Rice Research Institute (CRRI), Cuttack - 753 006, Odisha, India.
*Corresponding Author: Email- bharathkumar76@gmail.com

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Abstract- In this study, rainfed lowland rice variety, Swarna and semi-deep lowland rice variety Varshadhan with *Sub1* or without it were screened for submergence tolerance during seed germination and seedling stage. During seedling stage, the survival capacity was higher in Swarna-*Sub1* than Varshadhan-*Sub1* and however, the level expression of SUB1A gene was stronger in both cases. There was difference in the plant growth between them i.e. more in Varshadhan-*Sub1* when compared to in Swarna-*Sub1*. In case of cultivars with no *Sub1*, there was more survival and less growth rate in Swarna when compared to Varshadhan. In case of submergence tolerance during seed germination, no difference was observed in seed germination in both cultivars with *Sub1* or without it. But, there was difference in growth rate between Swarna and Varshadhan i.e. the growth rate was more in Varshadhan with *Sub1* or without it under submergence whereas in Swarna with *SUB1* was less and more in rice line with no *Sub1*. In gene expression analysis, expression of *RAmy3C*, *Adh1* and *Adh2* genes was found to be induce strongly in Varshadhan with *Sub1* and without it under aerobic and anaerobic condition as compared to Swarna. Thus, Varshadhan with *Sub1* has possessed both capacity to tolerate submergence during seed germination and seedling stage. This rice variety could be used by rice farmers who are facing problems by flash-flood at the time of seed sowing and transplanting in lowland and semi-lowland areas.

Keywords- gene expression, varshadhan, anaerobic condition, seed germination, submergence tolerance

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Introduction

Present and anticipated global food demands necessitate a significant increase in crop productivity in marginal farmlands. Rainfed lowland and deepwater rice together account for approximately 33 percent of global rice farmlands. About two thirds of the shallow and intermediate rainfed lowland rice lands are in India. Lowland rice is typically cultivated in paddies of 5 to 25 cm of standing water, which are highly vulnerable to monsoon flash floods of 50 cm or more that can rapidly and completely submerge plants [1]. Oftentimes, transient submergence is repeated or followed by a period of stagnant partial flooding. When partially or completely submerged, most rice varieties display a moderate capacity to elongate leaves and the portion of stems that are trapped underwater. This elongation growth leads to a spindly plant that easily lodges when floodwaters recede. If the flood is deep, underwater elongation growth can exhaust energy reserves, causing death within a matter of days. But, traditional varieties adapted to the submergence prone environments are low yielding due to their low tillering ability, long droopy leaves, susceptibility to lodging and poor grain quality. For example, rice variety with tolerance of submergence, Flood Resistance 13A (FR13A), was identified more than 25 years ago and tolerance in this landrace is controlled by the *SUB1* locus on chromosome 9 [2], which includes three ethylene response factor (ERF)-like genes (SUB1A, SUB1B, SUB1C) [3]. *Sub1* versions of popular rice varieties developed through the marker assisted backcrossing (MABC) approach [4,5] have displayed a high level of submergence tolerance compared to their parents because of retaining the desirable features of the original popular varieties. However, most of them are sensitive to stagnant flooding because of their short stature. At the same time, rice breeders successfully developed cultivars which have tolerance to stagnant flooding do not possess *SUB1* and they remain sensitive to short-term flash flooding.

Nowadays, new trend in rice production is marked with the shift from transplanting to direct seeding notably in areas where there is scarcity of irrigation water and high cost of labor. Compared to transplanted culture, direct seeding method can reduce labor input by as much as 90% and can shorten crop growth duration up to 14 days [6]. However, one of the important problems associated with direct wet-seeding is poor seedling establishment after sowing, which may be due to damages caused by anoxia by flooding during germination. Thus, rice plants are affected by four types of flood conditions such as flooding during germination, flash flood, stagnant flooding and deeper stagnant flooding depending on the plant traits and varietal types that are adapted to the conditions. In any particu-

lar field, more than one of these situations can occur in the same season or in different seasons. Therefore, it is preferable that varieties developed for flood-prone areas have a combination of tolerance traits when possible. In this study, we analyzed Varshadhan with *Sub1* or without it for submergence tolerance during seed germination and seedling stage along with a popular rainfed lowland rice variety, Swarna at phenotypic and genotypic level.

Materials and Method

Plant Materials, Submergence Screening and Anaerobic Seed Germination

In this study, *Sub1* and non-*Sub1* lines of Varshadhan and Swarna were used. For submergence screening, seeds of these lines were sowed in metal trays and seedlings were grown for 2-weeks. At 14-d-old seedling stage, metal trays were shifted to submergence tank and water level was raised to 90 cm height and water level was maintained for 2-weeks. Then, seedlings were de-submerged and the survival rate was recorded after 10 days [5]. For anaerobic seed germination, 25 grains of each genotype were dry seeded at about 1-cm depth in plastic tray containing finely ground field soil, followed by either normal watering (control) or flooding with about 10cm of tap water until the end of experiment. This greenhouse experiment was conducted using a randomized complete block design with three replicates [7]. Lengths of the shoot and root were measured in seedlings grown under either a control or in flooded soil, and plant survival was calculated based on the number of seedlings that emerged out from water. All measurements were made at 21-day after sowing. The number of seedlings emerging from the water surface was recorded and survival was calculated as the percentage of surviving seedlings relative to the number of seeds used.

RNA Extraction and Reverse Transcriptase (RT)-PCR

For gene expression analysis of *Sub1*A and *Sub1*C genes, leaf samples were collected from seedlings at 0 and 24h of submergence stress. A 100mg leaf tissue were ground in liquid nitrogen using mortar and pestle and total RNA was extracted using TRIzol according to manufacturer's instructions. cDNA sysnthesis was done using SuperScript™ III Reverse Transcriptase according to manufacturer's protocol (Invitrogen, California, USA) in a reaction mixture containing 50-75ng RNA with the final volume completed to 20µL using RNase free water. cDNA was synthesized at 50ºC for 60 min and PCR was done at annealing temperature of 59ºC using primer sequences of *Sub1*A and *Sub1*C. The primer sequence of Rubisco was used as loading control [5].

For expression analysis of ά-amylase, sucrose synthase, pyruvate decarboxylases and alcohol dehydrogenase genes under aerobic and anaerobic seed germination conditions, the experiment was done using petridishes (aerated) and test tubes (anaerobic) per genotype. For this experiment, Varshadhan and Swarna with *Sub1* or without it were used. Tissue sampling for RNA extraction was done at 24, 48 and 72 h after the start of seed incubation. Twenty dissected rice embryonic tissues with halves of the starchy endosperm removed (about 100 mg) were ground in liquid nitrogen using a mortar and pestle and total RNA was extracted using TRIzol according to manufacturer's instructions. cDNA sysnthesis was carried out using SuperScript™ III Reverse Transcriptase according to manufacturer's protocol (Invitrogen, California, USA) in a reaction mixture containing 50-75 ng RNA with the final volume completed to 20µL using RNase free water. The reaction mixture was run in the following conditions: cDNA synthesis was done at 50ºC for 1h followed by PCR amplification at 58ºC (annealing temperature) using primer sequence of *RAmy3C*, *RAmy3D*, *RAmy3E*, *Sus1*, *Sus3*, *Pdc1*, *Pdc2*, *Adh1* and *Adh2* genes [8]. Primer sequence of Actin was used as loading control.

Results

Evaluation for Submergence Tolerance

During Seed Germination

In this evaluation, the rate of seed germination was 100 percent in Swarna and Varshadhan with *Sub1* and without it under aerobic and anaerobic condition [Fig-1]. Under aerobic condition, the growth rate of Varshadhan line was recorded as follows: the higher rate in *Sub1* line (35.2cm) and lower rate in non-*Sub1* line (34.3cm) for shoot length; for root length, the higher rate in *Sub1* line (14.4cm) and lower rate in non-*Sub1* line (14.3cm). In Swarna line, the higher rate was recorded in non-*Sub1* line (26.3cm) and lower rate in *Sub1* line (26.2cm) for shoot length and for root length, the higher rate was noted in non-*Sub1* line (13.3cm) and lower rate was in *Sub1* line (13.0cm). Under anaerobic condition, the growth rate of Varshadhan was recorded as follows: the higher rate in *Sub1* line (24.1cm) and lower rate in non-*Sub1* (23.6cm) for shoot length; for root length, the higher rate in non-*Sub1* line (12.3cm) and lower rate in *Sub1* line (11.9cm). In Swarna, the growth rate was noted as follows: higher rate in non-*Sub1* line (17.9cm) and lower rate in *Sub1* line (13.9cm) for shoot length; for root length, higher rate in non-*Sub1* line (8.0cm) and lower rate in *Sub1* line (7.6cm) [Fig-2].

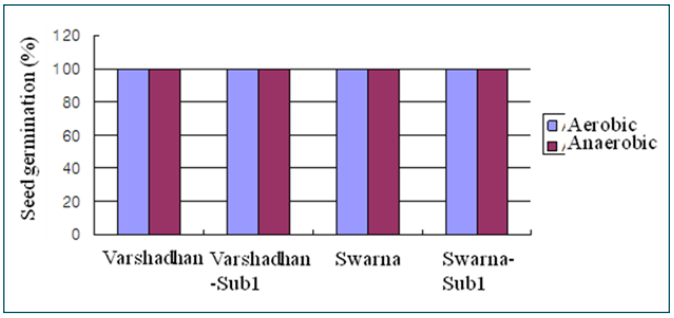

Fig. 1- Seed germination (survival) rate of Varshadhan and Swarna cultivars with SUB1 or without it under aerobic and anaerobic conditions after 21-days.

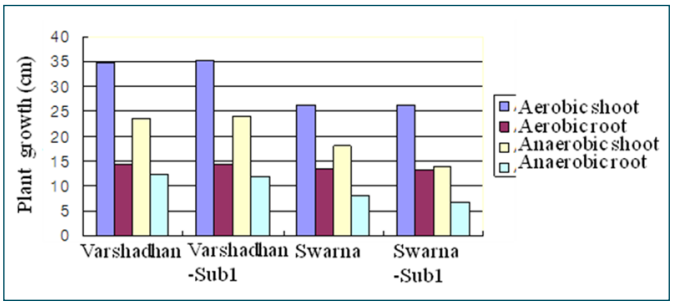

Fig. 2- Plant growth promotion of Varshadhan and Swarna cultivars with SUB1 or without it under aerobic and anaerobic conditions.

During Seedling Stage

In this evaluation, we recorded high survival rate in rice lines with *Sub1* (100 % in Swarna-*Sub1* and 78% in Varshadhan-*Sub1*) compared to rice line with no *Sub1* (5% in Swarna and 10% in Varshadhan) followed by submerging for 14-days [Fig-3]. During submergence, the rate of plant growth (leaf elongation) was higher in

rice lines with no *Sub1* (25.6% in Swarna and 30.4% in Varshadhan) than in rice lines with *Sub1* locus (8.4% in Swarna-*Sub1* and 11.5% in Varshadhan-*Sub1*) [Fig-4].

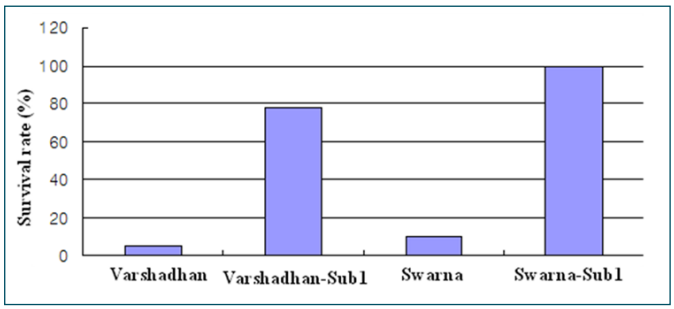

Fig. 3- Survival rate of Varshadhan and Swarna cultivars with SUB1 or without it after 14-days submergence stress during seedling stage.

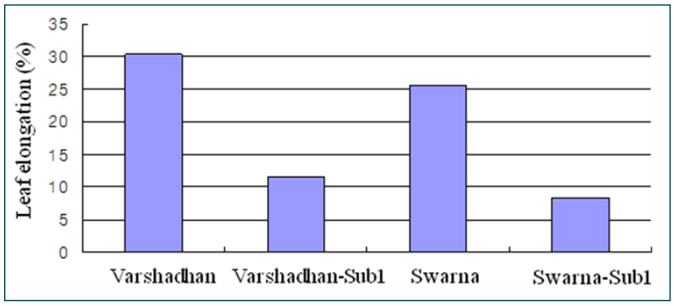

Fig. 4- Plant growth promotion of Varshadhan and Swarna cultivars with SUB1 or without it during submergence stress.

Gene Expression Analysis

During Seed Germination

During seed germination under aerobic condition, expression of *RAmy3C* gene was found to be induced strongly up to 72h in both *Sub1* and non-*Sub1* lines of Swarna and Varshadhan as well as 48h under anaerobic condition [Fig-5]. Expression of *RAmy3D* was induced only in *Sub1* line of Swarna but not in non-*Sub1* line under both conditions. In Varshadhan, its expression induced up to 72h in both *Sub1* and non-*Sub1* lines under aerobic condition but only at 24 h in non-*Sub1* line and at 48h in *Sub1* line. Expression of RAmy3E induced only at 72h in non-*Sub1* line of Swarna and its expression lost up to 72h in *Sub1* line under aerobic condition. Under anaerobic condition, no expression was found for *RAmy3E* in non-*Sub1* line of Swarna but its expression was at 48h in *Sub1* line. In case of Varshadhan, expression of *RAmy3D* was found to be induced gradually from 24h to 72h in both *Sub1* and non-*Sub1* lines under aerobic condition. Under anaerobic condition, its expression was only at 24h in non-*Sub1* line and up to 48h in *Sub1* line. Expression of Sus1 gene was detected to be strongly induced only at 48h in *Sub1* and non-*Sub1* lines of Swarna under both conditions. But its expression was noted up to 72h in *Sub1* and non-*Sub1* lines of Varshadhan under aerobic condition. Under anaerobic condition its expression declined at 24h in non-*Sub1* line and at 48h in *Sub1* line. Expression of Sus3 was found to be stronger up to 48h in *Sub1* line of Swarna under both conditions than non-*Sub1* line. In Varshadhan, its expression was stronger up to 72h in *Sub1* and non -*Sub1* line under both conditions. But, expression of Sus3 found to be declined at 24h in non-*Sub1* line and at 48h in *Sub1* line under both conditions. Expression of Pdc1was induced strongly at 72h in non-*Sub1* line of Swarna under aerobic condition and at 48h in *Sub1* line under anaerobic condition. In Varshadhan, its expression was stronger at 72h in non-*Sub1* line and at 24h in *Sub1* line under both conditions. Expression of *Pdc2* was induced gradually from 24h to 72h in non-*Sub1* line of Swarna and Varshadhan under aerobic condition as well as up to 48h in *Sub1* line under anaerobic condition. Expression of *Adh1* and *Adh2* genes was found to be induced strongly at 48h in *Sub1* and non-*Sub1* of Swarna under both conditions. In Varshadhan, their expressions were induced up to 72h in *Sub1* and non-*Sub1* lines under both conditions.

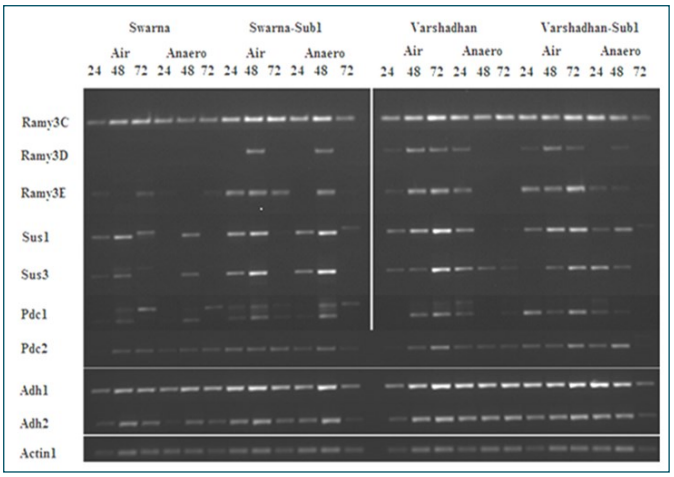

Fig. 5. Reverse-transcriptase (RT)-PCR analysis.

Gene expression of Amylases (*RAmy3C*, *RAmy3D* and *RAmy3E*), Sucrose synthases (*Sus1* and *Sus3*), Pyruvate decarboxylase (*Pdc1* and *Pdc2*) and Alcohol dehydrogenase (*Adh1* and *Adh2*) in Varshadhan, Varshadhan-Sub1, Swarna and Swarna-Sub1 under air and anaerobic treatments at 24, 48 and 72 h.

During Seedling Stage

Expression of SUB1A gene was detected to be induced strongly in *Sub1* line of Swarna and Varshadhan only at 24h. No induces of SUB1C gene was detected in both rice lines even at 24 h [Fig-6].

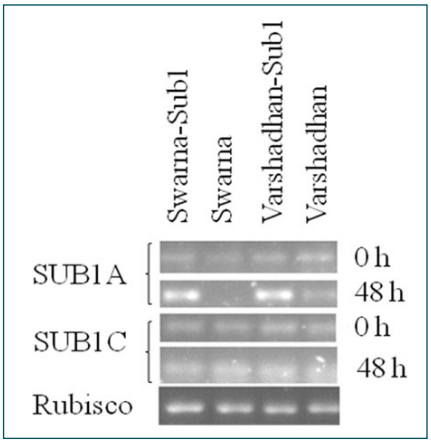

Fig. 6- Reverse transcriptase (RT)-PCR analysis.

Gene expression of SUB1A and SUB1C in Swarna-Sub1, Swarna, Varshadhan-Sub1 and Varshadhan at 0 h and 48h under submergence condition. Rubisco primer was used as loading control.

Discussion

In seed germination, we found no difference between rice lines with *Sub1* and without it of Varshadhan and Swarna under aerobic and

anaerobic condition. Under aerobic condition, the starch consumption rate is equal in both tolerant and sensitive genotypes and no difference can find between them. But, under anaerobic condition, the greater rate of reduction of starch concentration leads to faster seedling growth in tolerant genotype during the first 8 d of flooding whereas, in sensitive genotype, the starch consumption will be less [7]. In this study, there was difference in plant growth rate between *Sub1* and non-*Sub1* line of Swarna under anaerobic condition but not in aerobic condition. Supportively, in many previous studies, submergence tolerant genotypes (with *Sub1*) such as FR13A, M202, and *Nipponbare*, have been characterized as slow seed germinating genotypes under anaerobic condition [8, 13, 19]. Meanwhile, the process of plant growth in Swarna with no SUB1 did not affect. This result indicates that the suppression of growth in Swarna with SUB1 under flooding condition but not in Swarna with not SUB1 may associated with the elongation-suppressive effect of SUB1 and lead the seedlings not to elongate out of the water [10, 13]. In the case of Varshadhan, the rate of plant growth promotion was almost similar in both *Sub1* and non-*Sub1* lines under both conditions. But, there was difference only during seedling stage submergence in survival rate between rice line with SUB1 and without it. Thus, in Varshadhan, the expression of SUB1 did not interfere in shoot elongation during seed germination. Moreover, the expression of genes related to amylase (*RAmy*), sucrose synthase (*Sus*) and fermentation pathway (*Pdc and Adh*) was found to be induced strongly in Varshadhan genotype rather than Swarna under both conditions. According to Ismail, et al. [7], the high amylase activity is associated with faster rate of starch breakdown and higher soluble sugar concentrations in tolerant lines during flooding which presumably provided the substrates necessary for generating the energy required for growth and maintenance processes. Similarly, sucrose is another major energy source for germinating embryos and it is converted to hexoses under low-oxygen stress by sucrose synthase (*Sus*) [10]. Particularly, expression of *Adh1* and *Adh2* genes was strong and consistent in Varshadhan up to 72 h as compared to Swarna. Significantly, the alcohol fermentation pathway plays a dominant role under anaerobic conditions. Moreover, maintenance of cellular metabolism and function is reduced because of the low efficiency of anaerobic respiration. And also, induces of *Pdc* and *Adh* gene expression was found in these rice cultivars under aerobic condition and the result coincides with the previous report for the activity of *Pdc* and *Adh* under aerated conditions [11].

In the evaluation of submergence tolerance during seedling stage, higher survival rate was recorded in rice lines with *Sub1* locus (Swarna and Varshadhan) compared to rice lines without it after 14-days submergence stress. Contrast to the role of *Sub1*, the rate of plant growth was higher in rice lines with no *Sub1* when compared to rice lines with *Sub1* locus under submerged condition. These contrasting processes (Survival rate and plant growth) are due to very slow process of starch consumption in genotype with *Sub1* locus and rapid consuming of leaf starch and soluble sugars to maintain growth elongation in rice genotypes lacking *Sub1* locus during submergence [8]. However, there was difference between Swarna and Varshadhan with SUB1 in survival and growth rate as well as rice lines with no SUB1. In case of Swarna line, the survival rate was more and the growth rate was less as compared to Varshadhan line. This difference in survival and growth rate might associate with depletion of starch reserves depending on stature condition of plant. However, in gene expression analysis, the difference in the expression of SUB1A gene was in both Swarna and Varshadhan with SUB1. Likewise, there was no induces of SUB1C gene in both cultivars at 48h. The strong expression of SUB1A in rice lines with *Sub1* locus is associated with higher survival rate as well as less plant growth promotion. Moreover, the differential expression of *Sub1*A and *Sub1*C genes is linked with submergence tolerance and shoot elongation, respectively [8,12].

In the current study, the role of Varshadhan with SUB1 was highly significant in submergence tolerance during seed germination and seedling stage as compared to Swarna with SUB1. Even though Swarna-*Sub1* has been the most popular *Sub1* variety to date and has been well accepted by the farmers widely in eastern India, Bangladesh, and Nepal, the growth of Swarna-*Sub1* is greatly inhibited due to short stature when the water depth increases too fast [13]. Recently, Varshadhan (CRLC 899; IET 16481) is developed at the Central Rice Research Institute, Indian council of Agricultural Research, Cuttack from IR31432-8-3-2/IR31406-3-3-3-1/IR26940-3-3-3-1 and it is released for cultivation in waterlogged semi-deep areas (0-75cm) in eastern India during wet season. This variety is a photosensitive, non-lodging and yields 3.5-4.0t/ha. Also, it is moderately tolerant to neck blast, bacterial blight, sheath rot and white brown plant hopper. In addition, besides *Sub1* locus, higher activities of enzymes of the ethanolic-fermentation pathway (PDC and ADH) detected in Varshadhan with tall stature, might further improve the energy status [8,14,12,15]. Moreover, even though direct seeding is advantageous over transplanting in terms of labour requirement [16], unfortunately, very limited success has been achieved from previous efforts to improve the tolerance of genotypes for anaerobic conditions during germination since identified few elite breeding lines and genotypes in the initial screening still reduced in subsequent evaluation [17,18]. Therefore, Varshadhan with SUB1 can be used by rice farmers facing problems by flash-flood at the time of seed sowing and transplanting in rainfed lowland and semi-lowland areas.

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Conflicts of Interest: None declared.

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