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ORIGINAL ARTICLE

Structural Carbohydrates and Lignifications Associated with Submergence Tolerance in Rice (*Oryza sativa* L.)

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Role of structural carbohydrate content and lignifications in rice shoot were studied in three *indica* rice (*Oryza sativa* L.) cultivars namely FR13A (tolerant to submergence), IR42 (susceptible to submergence) and Sabita (adapted to medium depth, 0-50 cm stagnant flooding), varying degrees of tolerance to submergence under control and 8 days after submergence conditions. During submergence, Sabita and IR 42 accelerated the rate of stem and leaf elongation more than that of FR 13A. Submergence significantly reduces the cellulose and hemicelluloses content. The decrease was more pronounced in susceptible rice cultivar than that of tolerant rice cultivar. Lignifications' was monitored by measuring the content of lignin and the activities of two enzymes of the lignin biosynthetic pathway, coniferyl alcohol dehydrogenase (CAD) and phenylalanine ammonia-lyase (PAL) in rice shoots. Lignin content and PAL and CAD activity was more in susceptible cv. both under control and 8d after submergence. In conclusion submergence induced elongation of rice shoot might decreased the structural carbohydrate level as our experiment showed a significant negative correlation of cellulose and hemicelluloses with plant height but also positively associated with plant survival under submergence. The content of lignin and activities of CAD and PAL showed negative association with shoot elongation, yet the association of these parameters with survival was non-significant.

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The submergence due to water-logging *inter alia* various biotic and abiotic constraints resulting in partial to near complete crop damage and poor grain yield (Sarkar *et al*., 2006; Das *et al*., 2009). Submergence tolerance is a metabolic adaptation in response to anaerobiosis that enables cells to maintain their integrity, such that plant survives without major damage. Plants that survive or succumb to transient submergence differ in the timing and duration of carbohydrate consumption

and anaerobic metabolism (Fukao and Bailey-Serres, 2004; Fukao *et al*., 2011). Maintenance of high levels of stored non-structural carbohydrates in the seedlings prior to submergence coupled with minimum shoot elongation are all desirable traits for submergence tolerance (Das *et al*., 2009; Hattori *et al*., 2009). Deepwater rice responds to partial submergence by enhancing cell division and elongation in the internodal regions of underwater stems, via a mechanism triggered by entrapment of ethylene, which promotes abscisic acid (ABA) degradation and increases gibberellic acids (GA) and their downstream effects (Kende *et al*., 1998; Hattori *et al*., 2009). This unusually robust underwater elongation is controlled by three quantitative trait loci (QTLs). Of these, the SNORKEL QTL on chromosome12 encodes two ethylene responsive factor (ERF), DNA binding proteins, SNORKEL1 (SK1) and SNORKEL2 (SK2), that are absent from the non-deepwater rice accessions evaluated to date (Hattori *et al*., 2009). The underwater escape strategy of deepwater rice resembles that of semi-aquatic plants that are adapted to environments that incur prolonged shallow floods. A pronounced strong elongation growth maintains sufficient aerial tissue above the air–water interface for efficient photosynthesis and oxygen exchange with submerged organs (Bailey-Serres and Voesenek, 2008). A number of cell wall component and internodal growth are induced by partial submergence of deepwater rice (Kende *et al*., 1998). Lignin is the complex phenolic molecule second highest after cellulose nearly 30 % of organic carbon in the plant tissue (Rose-John and Kende, 1984). The synthesis of lignin is one of the most expensive biosynthetic processes in plants in terms of energy demand (Sauter and Kende, 1992). Lignification of transgenic Tobacco plants was paralleled by up- or down-regulation of the expression of lignin biosynthetic genes showed that altered biomass accumulation was brought about by modified lignin deposition that was associated with changes in expression of pathway-specific genes (Biemelt *et al*., 2004). Non-structural carbohydrate is the main source of fuel under submergence more numbers of investigations were carried with the role of non-structural carbohydrate and submergence tolerance. Studies related with structural carbohydrate content and submergence tolerance is scanty. Lignin biosynthesis was studied mainly with floating rice with longer partial submergence, however, no work has been done with non-floating rice cultivars under short period (1-2 weeks duration) of complete submergence. So the present investigation is undertaken to study the role of structural carbohydrates like cellulose, hemicelluloses and lignin with lignifications in relation to submergence tolerance in rice.

MATERIALS AND METHODS

Plant materials and Growth conditions:

The study was conducted by taking three *Indica* rice cultivars namely FR13A, IR42 and Sabita having differential response to flooding. FR13A, which is known to be tolerant to complete submergence and IR42, which is susceptible have been used in many physiological studies (Panda *et al*., 2006), Sabita (adapted to medium depth stagnant flooding, 0-50 cm), a local cultivar that elongates fast under complete submergence and survives if leaf tips remain above the water surface and it resemble likely elongating type under complete submergence (Sarkar *et al*., 2001).

The three cultivars were sown directly in earthen pots containing two kg of farm soil and farmyard manure in a 3:1 ratio. Each pot was

supplied with 88 mg urea, 190 mg single super phosphate (P_2O_5) and 50 mg murate of potash (K_2O). Ten days after germination, the seedlings were thinned and five plants per pot were maintained. The pots containing twenty one-days old seedlings were completely submerged in concrete tanks filled with water to a height of 110 cm. The plants were maintained under two treatments: (1) complete submergence for 8 d, (2) control growth condition i.e. without submergence treatment. The experiments were carried out in three replications and were statistically analyzed.

The characteristics of the floodwater in terms of light transmission were measured at 12:00 h (LI-COR, Lincoln, USA), and water temperature and oxygen concentration were determined at 06:00 and 17:00 h (Syland, Heppenheim, Germany) every alternate days. Light intensity at 60 cm water depth or at the vicinity of canopy level ranged from 205 to 310 µ mol m^{-2} s^{-1} whereas it was 1643 to 1712 µ mol m^{-2} s^{-1} above the water surface. The oxygen concentration at the same water depth was 2.6 to 3.2 mg L^{-1} at 06:00 h and 4.4 to 5.5 mg L^{-1} at 17:00 h. The temperature was being 28.2 ^{o}C to 30.4 ^{o}C throughout the period of experiment.

Estimation of Cellulose, hemicelluloses and lignin content:

Shoot cellulose content was estimated by taking 200 mg of dry powder with 3 ml of acetic-nitric reagent (150 ml of 80% acetic acid with 15 ml of concentrated nitric acid) and mixed in a vortex (Thimmaiah, 1999). The tubes were placed in water-bath for 30 min, cooled and then centrifuged for 15-20 min. The supernatant was discarded. The residue was washed with distilled water. Then 10 ml of 67 % H_2SO_4 was added and allowed to stand for 1 h. The above solution was diluted to 100 ml. One ml of diluted solution was mixed with 4 ml of anthrone reagent and glucose was estimated as described earlier. The amount of cellulose was calculated from the standard curve and expressed in mg cellulose g^{-1} dry wt.

Hemicellulose content was estimated according to the procedure of Loomis and Shull (1937). One g of dried powder was extracted at least twice in 20 ml of hot 80 % ethanol for 10 min. The supernatant was discarded each time; the residue left was dried at 70° C in an oven. The dried sugar free residue hydrolyzed with (1N) HCl in a 25 ml open conical flask for 1 h at 15 LbsPsi in an autoclave. The reducing sugar liberated was determined using anthrone reagent. The amount of sugar multiplied by 0.9 to obtain the amount of hemicellulose and expressed in mg hemicellulose g^{-1} dry wt.

Lignin was estimated according to the procedure of Thimmaiah (1999). For lignin estimation 100 mg oven dried sample was moisten in a mortar with water, grinded with ether until it was free from chlorophyll pigment. The sample was centrifuged at 2000 g for 5 min. Supernatant was discarded and the pellet was washed with water. The process was repeated twice. Two ml of NaOH was added to the residue and placed it for 12 -16 h at 70-80° C. The hot alkali extract (0.55 ml) was taken in a test tube containing 1-3 µg of phenol, 0.4 ml of (0.5 M) Tris-HCl (pH 9.0) buffer and 0.05 ml freshly prepared alcohol solution containing 25 µg of 2,6 – dichloroquinone chlorimide. After incubation for 1 h in the room temperature the absorbance was taken at 610 nm using guaiacol as a standard. By using the conversion factor 32 the lignin content was calculate (32 x mg phenol = mg lignin)

Measurements of Phenylalanine ammonia-lyase (PAL, EC. 4.3.1.5) and Conifferyl alcohol dehydrogenase (CAD, EC. 1.1.1.195) activity:

PAL and CAD activities were measured according

to the methods of Sauter and Kende (1992) by taking 500 mg of fresh plant tissue was homogenized in 5 ml of cold 25 mM borate-HCl buffer (pH 8.8) containing 5 mM marcaptoethanol and 1 % Polyvinylpyrrolidone and centrifuged at 12000 rpm for 20 min. The reaction mixture for assaying PAL contained 0.5 ml 100 mM potassium borate buffer (pH 8.8), 0.2 ml enzyme extract, 1.3 ml water and 1 ml L- phenylalanine (50 mM) and kept for 1 h for incubation at room temperature. The reaction was stopped by adding 10 % TCA and the absorbance was recorded at 290 nm. The activity was expressed µ mol trans–cinnamic acid formed h^{-1} mg^{-1} protein using cinnamic acid as standard.

The reaction mixture for the assay of CAD contained 1.8 ml tris-HCl (400 mM) buffer (pH 9.2), 0.5 ml coniferyl alcohol (15 mM), 0.5 ml NADP+ (0.28 mM) and 0.2 ml enzyme extract. The absorbance at 400 nm was monitored for 3min. The activity was expressed in change in OD min^{-1} mg^{-1} protein.

Statistical analysis

Differences between various parameters were compared by ANOVA using CROPSTAT (International Rice Research Institute, Philippines) software's least significant difference (LSD*P<0.05), as this is a good test for determining whether means were significantly different. Associations among different parameters were examined by simple correlation analysis using CROPSTAT software.

RESULTS AND DISCUSSION

Submergence or water logging imposes a complex abiotic stress on rice plant, affects numerous physiological and metabolic processes (Sarkar *et al*., 2006; Bailey-Serres and Voesenek, 2008; Jianxiong *et al*., 2010). Deepwater rice responds to partial submergence to complete submergence by enhancing cell division and elongation in the internodal regions of underwater stems and rapid cell expansion is necessary for fast elongation under flooding and low-oxygen stress (Rose-John and Kende, 1984; Kende *et al*., 1998). In this experiment the three cultivars responded differently to submergence in terms of survival and elongation. Ninety percent of FR 13A plants survived after 8d of submergence in comparison with less than 20 % in Sabita and 15 % in IR42 (data not shown). Plant height increased due to submergence in all the cv. (Table 1) but the elongation was greater in Sabita and IR42 compared to FR13A. After 8 d of submergence, the increase in plant height was more than 98 % and 95 % in Sabita and IR42 respectively whereas FR13A only increased by 34 %.

The levels of cellulose content in rice shoot were not significantly different among tolerant and susceptible rice cv. under control condition. Submergence significantly decreased the cellulose content in susceptible IR42 cv. as compared to air grown control plants but in FR13A and Sabita the cellulose content was not significantly changed (Table 1). Similarly the hemicelluloses content also significantly decreased under submergence in all the rice cv. studied compared to the control plant but the reduction was more pronounced in susceptible cv. IR42 (39 %) than Sabita (26 %) and FR13A (21 %). Lignin content in rice shoot was significantly more in susceptible cv. IR42 in compared to FR13A and Sabita and was significantly decreased due to submergence in all the cvs. (Table 1). The decreased was more pronounce in IR42 (30 %) than that of FR13A and Sabita (17 %). Internodes of deep water rice are induced to elongate rapidly

by partial submergence, or by treatment with ethylene or gibberellin. This growth response is based, in part, on enhanced cell elongation and an increase in the size of the internodal growing zone (Sauter and Kende, 1992; Hattori *et al*., 2009). For this, lignification is the process which limits the growth and elongation of internodes (Kende *et al*., 1998). Our experiment showed a significant negative correlation of structural carbohydrate viz. cellulose, hemicelluloses and lignin content of the rice shoot with plant height (Table 2). The results showed that submergence induced elongation of rice shoot decreased the structural carbohydrate. The result also showed that the shoot cellulose and hemicelluloses content exhibited significant positive association with plant survival and submergence tolerance (Table 2). Probably greater content of structural carbohydrate inhibited the elongation growth, restricted the use of non-structural carbohydrate content and indirectly helped in plant survival under complete submergence. Minimum elongation is a desirable trait for survival under complete submergence (Xu *et al*., 2006; Sarkar *et al*., 2006)

Submergence induced lignifications in rice shoot was monitored by the activities of two enzymes of the lignin biosynthetic pathway, coniferyl alcohol dehydrogenase (CAD) and phenylalanine ammonia-lyase (PAL). Conniferyl alcohol dehydrogenase (CAD) is an enzyme which is specific for the synthesis of lignin monomers and phenylalanine-ammonia lyase (PAL) is a highly regulated enzyme that plays a central role in the phenyl propanoid pathway. Its reaction product trans-cinnamic acid is a precursor in the synthesis of many secondary plant products including lignin (Sauter and Kende, 1992). PAL activity was significantly more in IR 42 and Sabita than FR13A both under control and 8d after submergence (Fig. 1 A). Submergence significantly decreased the PAL activity, which was more pronounced in IR42 (43 %), FR13A (37 %) and Sabita (20 %) than that of air grown control plants. CAD activity was significantly higher in susceptible cultivars IR 42 compared to Sabita and tolerant cultivar FR13A under control condition (Fig. 1 B). Submergence also significantly decreased the CAD activity and was more pronounced in FR13A (50 %), IR42 (40 %) and Sabita (28 %). Similar observation also reported by Sauter and Kende (1992) that in submerged rice plant, PAL and CAD activity was lower than that of air grown plants. The content of lignin and activities of CAD and PAL showed negative association with shoot elongation, yet the association of these parameters with survival was non-significant (Table 2). Sarkar *et al*. (1996) reported greater survival of plants under submergence even in more elongating rice cultivar provided the genotype possessed great quantity of non-structural carbohydrate before submergence. More work is needed to elucidate the role of these enzymes in plant survival under complete submergence.

In conclusion submergence induced elongation of rice shoot might decreased the structural carbohydrate content as our experiment showed a significant negative correlation of cellulose and hemicelluloses with plant height but also positively associated with plant survival under submergence. Lignin content and lignifications was more pronounced in susceptible cultivar to flooding stress. The process of lignifications in rice shoot might be inhibited by elongation induced by flash flooding/submergence but not associated with the plant survival.

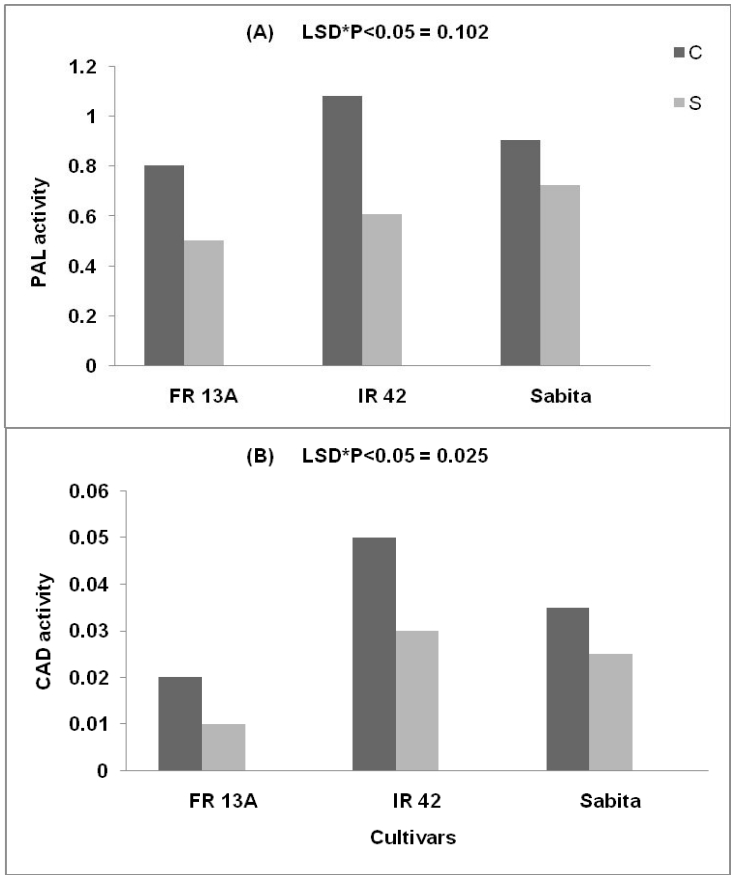

Figure 1: Lignin biosynthetic enzymes like phenylalanine ammonia-lyase (PAL, µ mol cinamic acid min^{-1} mg^{-1} protein) and Coniferyl alchohol dehydrgenase (CAD, Change in optical density min^{-1} mg^{-1} protein) activities in rice under control and 8 d after submergence. C: control, S: Submergence for 8 d.

Table 1: Structural carbohydrates like cellulose, hemicelluloses and lignin content and plant height in different rice cultivars before submergence (BS) and 8 days after submergence (AS). Data were presented as mean ± Standard deviation (n = 3).

Cultivars	Cellulose (mg g^{-1} dry wt)		Hemi cellulose (mg g^{-1} dry wt)		Lignin (mg g^{-1} dry wt)		Plant height (cm $plant^{-1}$)	
	BS	AS	BS	AS	BS	AS	BS	AS
FR 13A	230.4±6.5	225.5±3.5	138.4±5.3	108.5±3.4	1.84±0.09	1.52±0.08	31.5±1.2	42.3±1.1
IR 42	226.5±8.2	195.6±5.3	132.5±6.2	80.6±4.2	2.75±0.12	1.90±0.13	26.9±2.3	52.5±2.1
Sabita	236.4±7.3	227.5±7.3	127.5±8.3	93.2±4.3	2.35±0.11	1.95±0.11	30.2±1.5	58.3±1.3
LSD*P<0.05	16.8		12.5		0.25		2.8	

Table 2: Association of different structural carbohydrate contents and lignin biosynthetic enzymes phenylalanine ammonia-lyase (PAL) and coniferyl alchohol dehydrgenase (CAD) with plant height and survival (%) in different rice cultivars. *, Significance at P< 0.05; **, Significance at P< 0.01; ns, non-significant.

Parameters	Plant height	Survival
Cellulose	-0.543**	0.694**
Hemi cellulose	-0.923**	0.924**
Lignin	-0.548**	0.294^{ns}
PAL	-0.681**	0.406^{ns}
CAD	-0.491*	0.108^{ns}

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