

Original Research Article

EFFECT OF TRANSPLANTING TIMES ON PHENOLS, ENZYME AND CARBOHYDRATES IN RICE GENOTYPES

Abstract

The present study was conducted to understand the biochemical changes in rice (*Oryza sativa* L.) genotypes viz., heat tolerant (N22), moderately heat susceptible (IR8) and fine grain rice variety (PR116) in response to different transplanting time. Constitutive level of phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL) was found to be significantly higher in N22 than in IR8 at very early (D1) and early transplanting (D2) times. Heat stress caused a significant increase in the level of the phenolics constituents. Total free amino acid were significantly higher in N22 than in PR116 and IR8. Total sugars and reducing sugars decreased at D1 and maximum content was found in IR8. The starch content was found to be maximum in N22. Total amino acid content showed an increase with increase in heat stress in IR8 and PR116. Higher expression of PAL, TAL and phenolic constituents in N22 than in the IR8 and PR116 is suggestive of their significant role in development of tolerance exhibited by the cultivar N22 under heat stressed conditions.

Keywords: Rice, Transplanting Times, Heat Stress, Antioxidants, Phenols.

1. INTRODUCTION

Rice is the most important staple food crop grown in the areas where temperature is optimum for rice production and heat stress may limit sustainable rice production in these areas [1]. An environmental parameter that is highly variable, over various time scales, is ambient temperature. High temperatures can cause various problems for cellular functioning and thereby affecting plant fitness in the longer term. As a consequence of global warming, hot days and heat waves are predicted to increase both in frequency and in intensity in many temperate regions in the coming decades [2]. High temperature is a limiting factor that affects plant growth and crop yield in tropical and sub-tropical areas. The increasing global temperature will change the optimum conditions for crop production and thereby affecting agriculture. High temperature stress is a great modulator of growth and productivity [3]. Heat stress reduces crop yield and affect plant's life cycle. There are also indications that heat causes oxidative stress and the

possible involvement of reactive oxygen species (ROS) in the prevention of damage by heat stress in crops such as rice, pepper and maize, which differ in their tolerance [4, 5, 6]. Rachel and Dolan [7] also observed that ROS are necessary for growth of root hair, where they manage the activity of Ca^{2+} channels necessary for primary growth.

Plants have a battery of enzymatic and nonenzymatic antioxidants to cope with continuous ROS production, which function as an extremely efficient cooperative system. An important first line in plant defence against infection is provided by the very rapid synthesis of phenolic compounds and their polymerisation of cell-wall [8]. The plant phenolics confer various physiological functions for plants to survive and to adapt to environmental disturbances [9]. These are diverse secondary metabolites (flavonoids, tannins, lignin etc) being present in abundance in plant tissues [10]. Flavonoids are able to alter peroxidation kinetics by modifying lipid packing and decreasing fluidity of membranes, which sterically obstruct free radical diffusion and thereby restrict peroxidative reactions [11, 12]. This property of flavonoids underlies its antioxidant properties. Phenolic compounds are also involved in hydrogen peroxide scavenging cascade in plant cells [13].

Both phenolic compounds and flavonoids are powerful antioxidants that can act as free radical scavengers [14, 15], reducing agents, and/or metal ion chelators [16], thus providing various human health benefits [17].

The mechanisms leading to the survival of a crop under heat stress entail changes in physiology and accumulation of osmolytes like proline, glycinebetaine, soluble sugars and proteins [18, 19]. Soluble sugar also plays important roles in osmotic regulation of cells under heat stress [20].

The objectives of this study were to determine phenolic compound content, including polyphenols and flavonoids in rice genotypes during their reproductive phase of growth when transplanted at different times.

2. MATERIALS AND METHODS

Three rice genotypes viz. heat tolerant N22, heat susceptible IR8 and PR116 were employed in this study. The rice genotypes were sown during mid-March (very early), mid-April (early) and mid-May (normal). The rice seedlings of these cultivars were raised in the wooden trays (50 x 40 x 10 cm) in the glasshouse. Thirty-day old seedlings were transplanted and then the activities of the enzymes were studied in the flag leaf during anthesis and dough stage of grain-filling stage. The recommended agronomic practices were followed. The temperatures under the three sowings were recorded by the field meteorological laboratory and then the activities of the enzymes were studied in the flag leaf during anthesis and dough stage of grain development. All enzymatic and other estimations were estimated in the mid-region of the flag leaf.

2.1 Collection of samples

Flag leaf samples were collected at two different stages viz. anthesis and dough stages. Leaf blades were cut with the help of a scissors from the rice seedlings at each stage, immediately put in properly labelled polyethylene bags and then placed in the ice box. Each sample was divided into two parts. The one part was stored in 80 per cent ethanol in a deep-freezer for the estimation of carbohydrate content while the second part was air dried and then dried to a constant weight in an oven at $50 \pm 2^\circ\text{C}$. This part of the sample was used subsequently for the estimation of total phenols, *ortho*-dihydroxyphenols and flavonols.

2.2 Extraction and estimation of enzymes

2.2.1 Extraction

10 mg of leaf sample was extracted with 5 ml of ice cold 0.1 M Tris HCl buffer, pH 7.5, containing 5 mM β -mercaptoethanol using ~~pre-chilled~~ pestle and mortar. The homogenate was centrifuged at $10,000 \times g$ at 4°C for 25 minutes and the clear supernatant was used for estimating the enzymes- polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL).

2.2.2 Estimation of Polyphenol oxidase

The enzyme was assayed by monitoring the Intensity of dark coloured polymeric compound formed from catechol. 2.5 ml of 0.01 M catechol (in 0.1 M phosphate buffer, pH 6.0) was taken in the cuvette and absorbance was set at zero. Then 0.4 ml of enzyme extract was added to catechol solution and the increase in absorbance was recorded at 495 nm after every 30 seconds upto 3 minutes [21]. Enzyme activity was expressed as the change in absorbance $\text{min}^{-1} \text{g}^{-1}$ fresh weight and $\text{min}^{-1} \text{mg}^{-1}$ protein.

2.2.3 Estimation of Phenylalanine ammonia lyase

The enzyme was assayed by following the appearance of *trans*-cinnamic acid resulting from deamination of L-phenylalanine. The reaction mixture contained 2.5 ml of 0.03M phenylalanine in 0.05 M sodium borate buffer (pH 8.8) and 0.2 ml of enzyme extract. The reaction mixture was incubated at 37°C for 1 hour. The reaction was terminated by addition of 0.3 ml of 5N HCl. The absorbance was recorded at 290 nm against water blank [22]. The concentration of *trans*-cinnamic acid was read from the standard curve prepared by using *trans*-cinnamic acid in the range of 5-40 μg . The enzyme activity was expressed as μg *t*-cinnamic acid produced $\text{h}^{-1} \text{g}^{-1}$ fresh weight and $\text{h}^{-1} \text{mg}^{-1}$ protein.

2.2.4 Estimation of Tyrosine ammonia lyase

The enzyme was assayed by following the appearance of *trans p*-coumaric acid resulting from the deamination of L-tyrosine. The reaction mixture contained 1.0 ml of 3% tyrosine prepared in 0.05 M sodium borate buffer (pH 8.8), 0.4 ml of enzyme extract, and 1.0 ml of 0.05 M sodium borate buffer (pH 8.8). The reaction mixture was incubated at 37°C for one hour. The reaction was terminated by the action of 0.1 ml of 5N HCl. The absorbance was recorded at 310 nm against water blank [22]. The concentration

of *p*-coumaric acid was read from the standard curve prepared by using *p*-coumaric acid in the range of 5-40 µg. The enzyme activity was expressed as µg *p*-coumaric acid produced h⁻¹ g⁻¹ fresh weight and h⁻¹ mg⁻¹ protein.

Total soluble protein content of the enzyme extract was determined using BSA as a standard [23].

2.3 Extraction and estimation of carbohydrates

Total sugars were extracted from the sample stored in 80 % ethanol followed by 70 % ethanol. From the pooled extract, total sugars were estimated (Dubois *et al* 1956). To  of appropriately diluted sugar extract, containing 20-50 µg of sugars 1 ml of 5% phenol was added followed by addition of 5 ml of concentrated sulphuric acid. The sulphuric acid was poured directly in the centre of test tube to ensure proper mixing of the solutions. After 30 minutes, the absorbance was measured at 490 nm against a reagent blank. The concentration of total sugars (as glucose) was calculated from glucose standards (10-100 µg) run simultaneously. The sugar free residue was dried at 60° C and used for the estimation of starch [24]. The amount of starch was calculated by multiplying the content of total sugars by a factor of 0.9. Reducing sugars were also estimated by the method of Nelson [25].

2.4 Extraction and estimation of total amino acids

Total free amino acids were estimated by method of Lee and Takahashi [26]. The sample was refluxed successively with 15 ml each of 80% and 70% ethanol, respectively on a boiling water bath for 15 min. The supernatants were pooled and filtered through Whatman paper No.1. The filtrate was made free from ethanol by evaporation on a flash evaporater. The aqueous extract was diluted to a volume of 25 ml with distilled water. To a known volume (1 ml) of the aqueous extract, 5 ml of ninhydrin reagent D (It was prepared fresh by mixing 1% Ninhydrin, Pure Glycerol and 0.5M Citrate buffer (pH 5.5) in a ratio of 5:12:2 (v/v/v)). was added. The mixture was gently shaken and kept for 10-15 min in a boiling H₂O bath. After cooling, absorbance of the colour developed was measured at 570 nm using L-Glycine (2-20 µg) as a reference standard amino acid.

2.5 Extraction and estimation of phenolic constituents

The content of total phenols and flavonols were determined by the method of Swain and Hills [27] and Balabaa *et al* [28] respectively.

40 mg of ground dried leaf sample was refluxed with 5 ml of 80% aqueous methanol for 1 hr at 80°C on water bath. The refluxed material was filtered through Whatman filter paper No. 1 and the volume was made to 10 ml by washing with 80% methanol. The extract thus prepared was used for the estimation of phenolic constituents viz. total phenols, *o*-dihydroxyphenols and flavonols.

 known volume of methanolic extract was evaporated to dryness and the residue was dissolved in 6.5 ml of distilled water. To this 0.5 ml of Folin-phenol reagent was added and shaken thoroughly. After five minutes, 1 ml of saturated solution of sodium carbonate was added. After one hour, absorbance of the

blue color developed was read at 760 nm against the reagent blank. Concentration of total phenols was determined from the standard curve prepared by using gallic acid in the range of 10-50 µg

2.5.1 Estimation of *o*-dihydroxyphenols

The methanolic extract was evaporated to dryness and the residue left behind was dissolved in 1 ml of distilled water. To this 0.3 ml of 10% TCA, 1 ml of 10% sodium tungstate, 0.5 ml of 0.5 N HCl and 1 ml of freshly prepared 0.5% sodium nitrite was added. A yellow colouration developed. After five minutes 2 ml of 0.5 N NaOH was added. The light cherry colour developed was read after 15 minutes at 540 nm against the reagent blank [29]. The concentration of *ortho*-dihydroxyphenols was read from the standard curve prepared by using catechol in the range of 6- 40 µg.

2.5.2 Estimation of flavonols

1 ml of methanolic extract was evaporated to dryness. The residue left behind was dissolved in 5 ml of 0.1 M methanolic solution of aluminium chloride. The yellow colour so developed was read at 420 nm against the reagent blank. The concentration of the flavonols was read from the standard curve prepared by using rutin in the range of 40-200 µg.

2.6 Statistical Analysis

Data were reported as mean ± standard deviation for triplicates determinations of each sample. The data was analyzed statistically by analysis of variance. For significant mean effects, means were separated using least significant test (LSD)

3. RESULTS AND DISCUSSION

The present study investigated the comparison of defence related enzymes and various metabolites in heat tolerant (N22), moderately heat susceptible (IR8) and fine grain quality (PR116) genotypes in response to different transplanting time.

The different sowing times selected in this study resulted in exposure of plants to varied temperatures at different stages of plant growth (Fig. 1)

During our study, the mean maximum temperature encountered during anthesis by N22 and IR8, PR116 was 3.7°C and 3.6°C higher respectively in early transplanting (D2) compared to normal transplanting (D3). During dough stage, the temperature rise observed was 4.4°C and 4°C in N22 and IR8 respectively (Fig. 1) at D2 compared to D3.

The temperature encountered by all the three cultivars at different transplanting time during anthesis was the same, but during dough stage, 1°C higher temperature was encountered by N22 at the normal transplanting date (D3) as compared to IR8 and PR116. It is clear from the temperature data that very

early transplanting (D1) and early transplanting (D2) resulted in exposure of crop to a higher temperature than the optimal temperature.

The activity of various defence-related enzymes viz. phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL), polyphenol oxidase (PPO) and non enzymatic contents namely, phenols, flavonols, carbohydrates and amino acid were studied in the flag leaf of rice varieties viz., N22 (heat tolerant), IR8 (moderately susceptible) and PR116 (fine grain rice variety) at different transplanting time were analysed at anthesis and dough stages of plant growth.

The oxidation of phenols is mediated by PPO and PO [30]. More specifically, PPO and PO have been related to the appearance of physiological injuries caused in plants by thermal stress [31, 32]. In our study, negligible amount of PPO activity was expressed in all the three cultivars at the anthesis and dough stages (Not given). Highest PPO activity in N22 was obtained at D1 of both the anthesis and dough stage while it was highest at D3 in the anthesis stage of both IR8 and PR116.

The activity of PAL increases in response to thermal stress and is considered to be one of the main components of cell acclimation against stress in plants [33]. PAL deaminates phenylalanine to form cinnamic acid, a key step in the synthesis of phenols and lignin. Maximum activity of PAL was detected in N22 and it decreased steadily from D1 to D3 in the both anthesis (0.83 to 0.39 *t*-cinnamic acid formed min⁻¹ mg⁻¹ of protein) and dough stages (0.69- 0.39 *t*-cinnamic acid formed min⁻¹ mg⁻¹ of protein) (Fig. 3).

A similar trend of PAL activity was observed in IR8 and PR116. The PAL activity was found to be minimum in IR8 (0.03 *t*-cinnamic acid formed min⁻¹ mg⁻¹ of protein) at the anthesis stage during D3. An intermediate response was observed in PR116. PAL activity has been reported to increase in response to thermal stress and is considered to be involved in cell acclimation against heat stress in plants [33].

Maximum TAL activity was found at D1 in the anthesis stage of PR116 (1.09 µg-*p*-coumaric acid formed min⁻¹ mg⁻¹ of protein) but the overall TAL activity was present in N22 (0.43 µg-*p*-coumaric acid formed min⁻¹ mg⁻¹ of protein) followed by PR116 (0.40) and IR8 (0.29 µg-*p*-coumaric acid formed min⁻¹ mg⁻¹ of protein). It decreased gradually from D1 to D3 and was higher in the anthesis stage compared to the dough stage (Fig. 3).

PAL and TAL are inducible in response to biotic stress such as chitin from fungal cell walls and abiotic cues [34]. It has been suggested that accumulation of soluble phenolics occur under heat stress which is accompanied with increased PAL and decreased PO and PPO activities [34].

Content of phenolic constituents in heat tolerant (N22), moderately heat susceptible (IR8) and fine grain rice variety (PR116) at different transplanting time

Total overall phenol content was highest in N22 (145.00 mg g⁻¹ fresh weight) and was found to be maximum at D2 in the anthesis stage (Fig.4). The minimum phenol content was present in IR8 (74.67 mg

g⁻¹ fresh weight). In general, total phenol content decreased from D1 to D3 with an exception at the anthesis stage in N22, where it was statistically the same at D1 and D2.

Flavonols constitute one of the major six subgroups of flavonoids found in higher plants [35]. Flavonoids and flavonoid derivatives play an important role in the protection against UV radiation and plant defence responses [35, 36]. In our study, overall content of flavonols was found to be the highest in N22 (25.64 mg g⁻¹ fresh weight) followed by PR116 and IR8. Maximum flavonol content was present at the anthesis stage of D1 and D2 in N22 while the minimum content was present at the anthesis stage of IR8 at D1 (Fig.5). Flavonoids are reported to perform the role of antioxidants, such as ASH and α -tocopherol [37]. They serve as ROS scavengers by locating and neutralizing radicals before they damage the cell and can thus help the plants under adverse environmental conditions [38]. Many flavonoid biosynthetic genes are induced under biotic and abiotic stresses, such as wounding, drought, metal toxicity and nutrient deprivation [35].

o-dihydroxyphenols content was maximum in N22 (13.54 mg g⁻¹ fresh weight) followed by PR116 (8.29 mg g⁻¹ fresh weight) and IR8 (6.68 mg g⁻¹ fresh weight) and was found to be higher in the dough stage compared to the anthesis stage. In N22, its content was highest at D3 (14.79 mg g⁻¹ fresh weight) and similar trend was found in IR8 and PR116 (Fig. 6).

Content of carbohydrates, amino acids and crude protein in heat tolerant (N22), moderately heat susceptible (IR8) and fine grain rice variety (PR116) at different transplanting time

Carbohydrates are a major category of compatible solutes that include hexoses (mostly fructose and glucose), disaccharides (sucrose, trehalose), sugar alcohols (inositol, mannitol) and complex sugars (raffinose and stachyose), all of which accumulate during stress [39, 40]. Soluble sugar plays important roles in osmotic regulation of cells under heat stress [41]. In our study, the overall total sugar content was statistically at par in N22 and PR116 while it was found to be the highest in IR8. Heat stress significantly decreased the total soluble sugar content in all the cultivars under study except the dough stage of PR116 at D2 when it was higher than at D3 (Fig: 7). Reducing sugar content was also decreased with heat stress and was found to be the lowest at D1 in all the three cultivars. The reducing sugar content was at par in N22 and PR116 and the minimum amount was present at the anthesis stage in N22 at D1. Higher content of reducing sugars was present at the dough except at D1 in PR116 and IR8 (Fig. 8). The overall starch content was maximum in N22 followed by PR116 and IR8. Highest content of starch was present at D3 followed by D2 and D1 while the overall starch content was higher in the anthesis than in the dough stage (Fig: 9). Accumulation of these sugars in chilling-resistant plants during winter has been reported and such accumulation appears to be conferring cryoprotective and osmotic regulatory properties [42].

The overall total amino acid content was significantly higher in N22 followed by IR8 and PR116 and the maximum amount was present in the anthesis stage of N22 at D3. The minimum total amino acid content was present in the dough stage of PR116 at D3 (Fig: 10). The total amino acid content varied with the cultivar/ variety, date of transplanting and also with the stage of sampling. No general trend could be discerned from the amino acid content.

CONCLUSION: It is clear from the above results that the high temperature tolerant variety N22 showed an increased activity of PAL and TAL under very early and early transplanting times. This underlines the protective role played by the antioxidant enzymes and the enzymes of phenylpropanoid pathway under heat stress and their significance as the basis of the tolerance exhibited by the tolerant cultivar to high temperature stress under early transplanting conditions.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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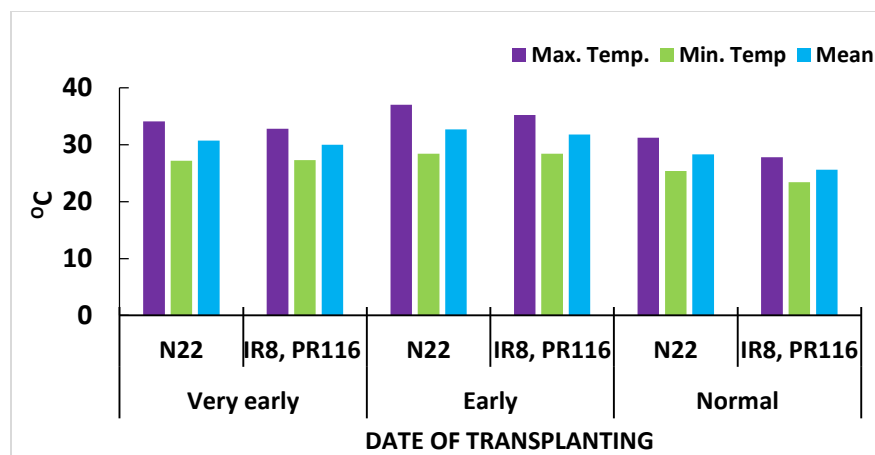


Fig.1 Temperature Profile

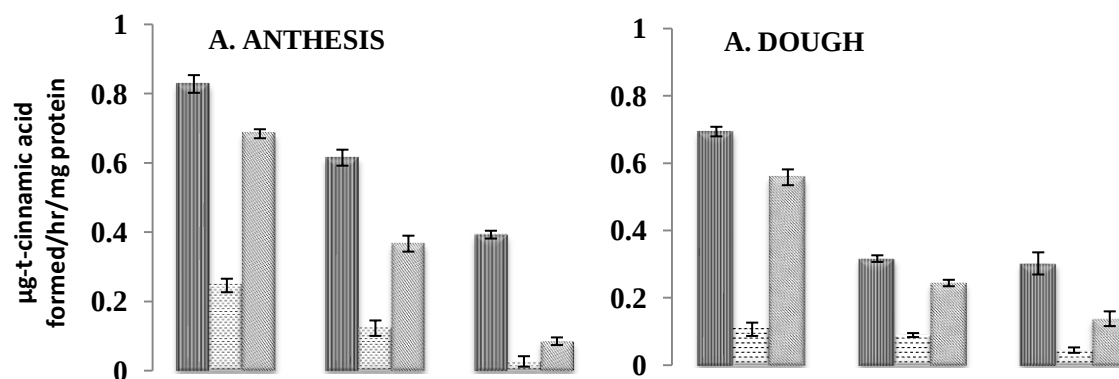


Fig 2: Effect of different dates of transplanting on the activity of Phenyl ammonia lyase (PAL) in different rice genotypes.

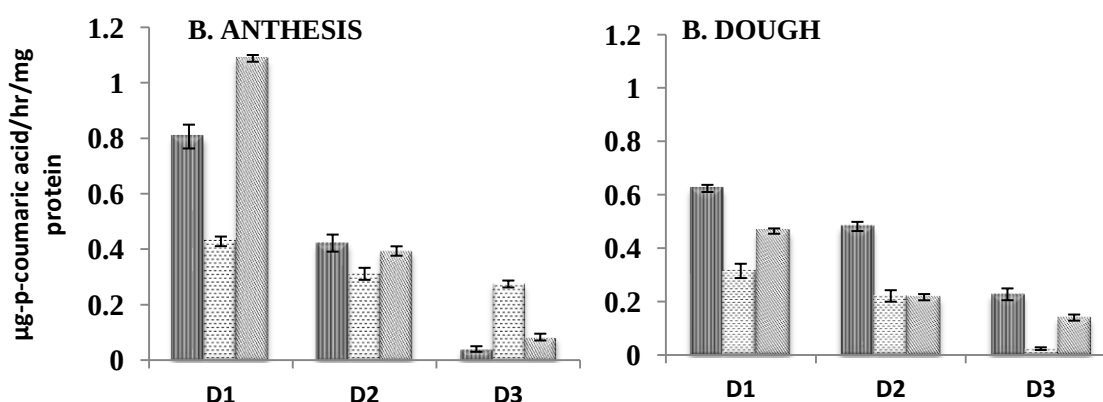


Fig 3: Effect of different dates of transplanting on the activity of Tyrosine ammonia lyase (TAL) in different rice genotypes.

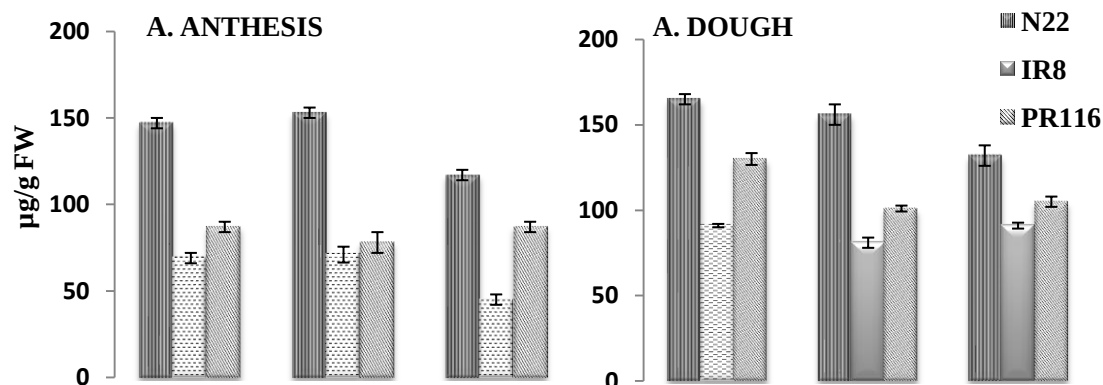


Fig 4: Effect of different dates of transplanting on Total phenols in different rice genotypes.

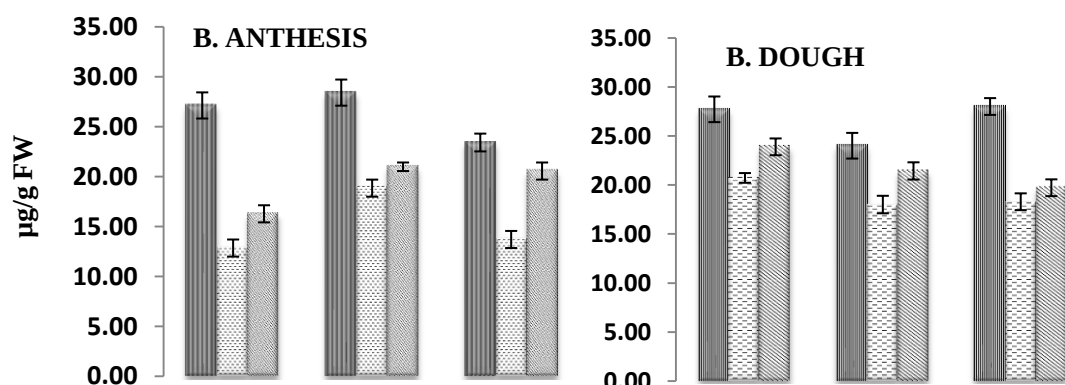


Fig 5: Effect of different dates of transplanting on Flavonols in different rice genotypes.

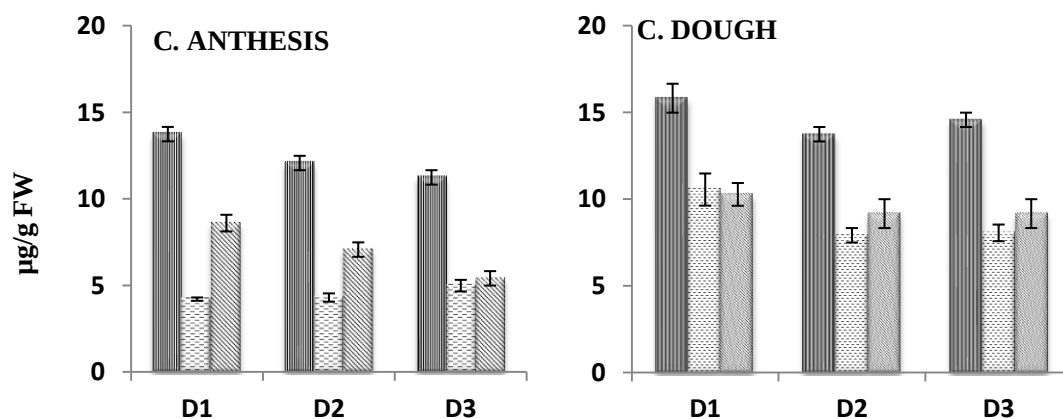
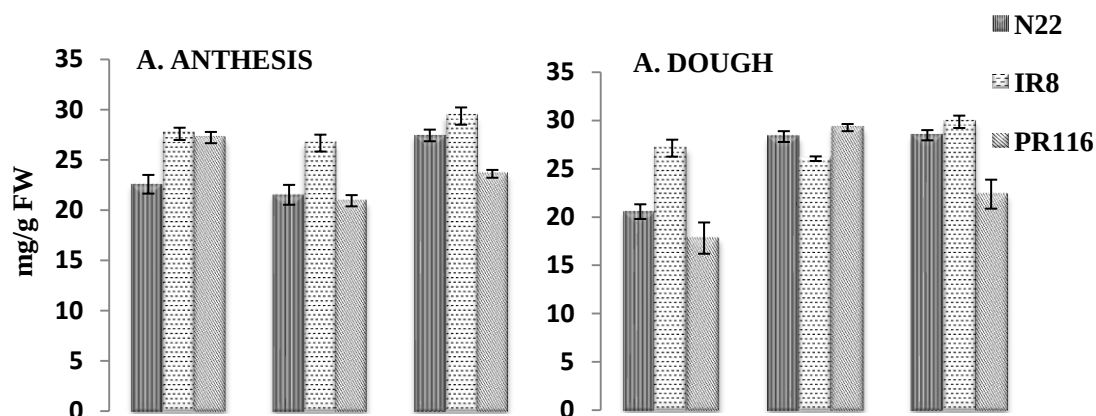


Fig 6: Effect of different dates of transplanting on *o*- dihydroxy phenols in different rice genotypes.
D1= very early transplanting (mid- April), D2= early transplanting (mid- May) and D3= normal transplanting (mid-June).

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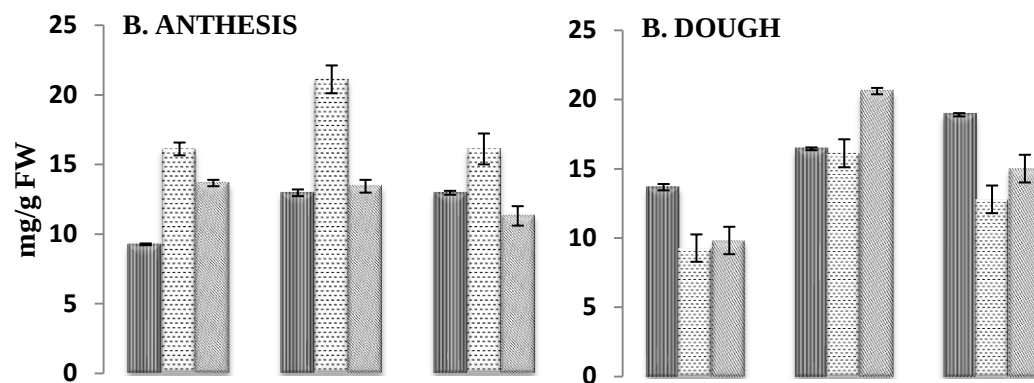


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372 **Fig 7: Effect of different dates of transplanting on the activities of A. Total sugars, B. Reducing**
 373 **sugars and C. Starch in different rice genotypes.**

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377 **Fig 8: Effect of different dates of transplanting on the Total sugars in different rice genotypes.**

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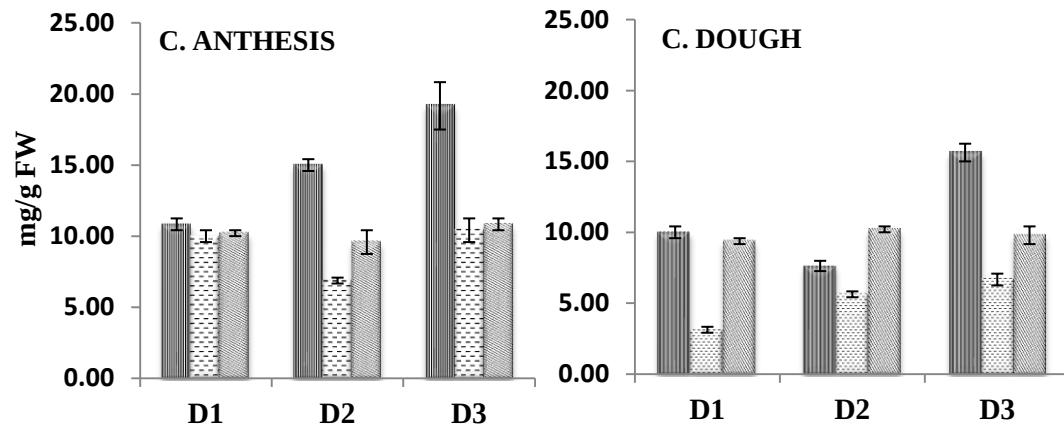


Fig 9: Effect of different dates of transplanting on the Reducing sugars in different rice genotypes.

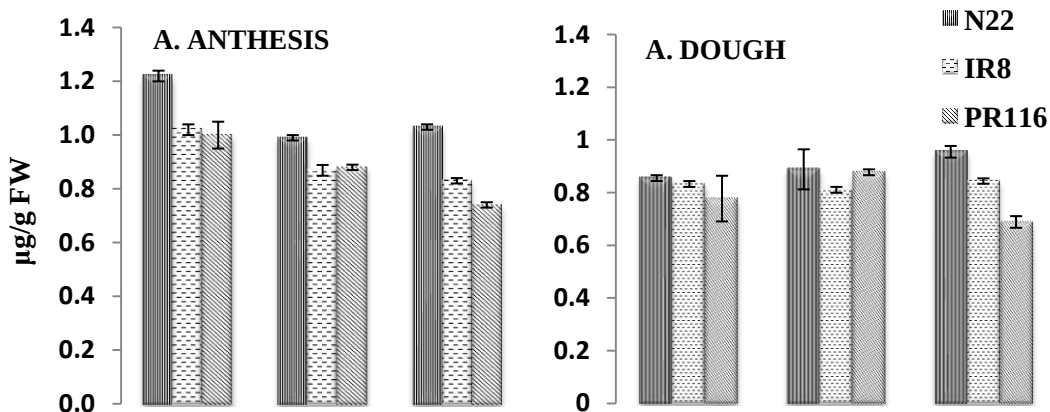


Fig 10: Effect of different dates of transplanting on Total amino acids in different rice genotypes.

D1= very early transplanting (mid- April), D2= early transplanting (mid- May) and D3= normal transplanting (mid- June).