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# AN EVALUATION OF WATER DEFICIT TOLERANCE SCREENING IN PIGMENTED INDICA RICE GENOTYPES

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**Abstract**

Eight pigmented genotypes of *indica* subspecies were geminated and then treated by mannitol-induced water deficit stress. A change of growth characteristics, photosynthetic pigments, lipid peroxidation, DNA content, proline content and anthocyanin accumulation in stressed seedling (100 mM mannitol) and control plant (0 mM mannitol) were calculated. Growth performances, photosynthetic pigment concentrations, and DNA contents in all rice genotypes were dropped whereas proline, anthocyanin contents and the lipid peroxidation levels were enriched. The stabilization in total photosynthetic pigment concentrations of stressed-seedlings were positively correlated to the proline or anthocyanin accumulation. In contrast, MDA content, the increases in the percentages of drought-stressed seedlings were negatively correlated to the proline or anthocyanin accumulation. The changes in biochemical, physiological and growth parameters were subjected to Ward's cluster analysis for water deficit tolerance. These cultivars could be classified into two groups, water deficit sensitive, SY, KD, KLD and TD49 and water deficit tolerance, KS, KK1, KK2 and BSR.

## Introduction

Water deficit is one of major abiotic stresses to reduce crop production worldwide, limiting the productivity of crop species, especially in arid and semiarid zones (>1.2 billion hectares) (Chaves & Oliveira, 2004; Kjine, 2006; Passioura, 2007). Crop improvement for water deficit tolerant trait is a fruitful topic to solve the dry land, especially rice crop (Ndijiondjop *et al*., 2010; Guan *et al*., 2010). Biochemical, physiological and growth changes in water deficit stressed plants have been well established. For example, plant accumulates organic solutes i.e. soluble sugar, proline and/or glycine betaine in responses to water deficit stress. These organic solutes function as osmoregulation defense mechanism localized in the cytoplasm, leading to maintain the turgor pressure of the cell when subjected to water deficit stress (Yamada *et al.*, 2005). Proline enrichment in the stressed plants is a general responses to various abiotic stresses, hence it has been developed as effective indices for stress tolerance identification (Abdel-Nasser & Abdel-Aal, 2002; Akram *et al.*, 2007). Moreover, reactive oxygen species (ROS) production has been reached when plant subjected to water deficit stress. Enrichment of ROS directly exhibits the oxidative damage especially constituent change of unsaturated fatty acids, leading to alter the membrane structure and their properties (Quan *et al.*, 2004). The reduction of oxidative damages using antioxidant system is generally reported in stressed-plants (Kranner *et al.*, 2002). Anthocyanin, a member of flavonoid components, is well known as powerful hydrogen-donating antioxidants and ROS scavengers (Rice-Evans, 2001). There are some reports to exhibit the anthocyanin in higher plants when subjected to different abiotic stresses, salt stress, water deficit, cold and ultraviolet-B irradiation (Bednarek *et al.*, 2003; Chutipaijit *et al.*, 2009).

Plant biochemical and physiological changes in many crop species in response to water deficit have been implemented as effective indices for selection in plant breeding programs (Ashraf & Foolad, 2007; Ashraf, 2010). In large population of breeding lines, a rapid screening is very important process to pyramiding the best genotype using effective criteria for water-deficit tolerance. Some previous studies suggested that multivariate cluster analysis is practically required for water-deficit classification in rice breeding programs (Cabuslay *et al.,* 2002; Cha-um *et al*., 2010). Rice (*Oryza sativa* L.) is a major global foodstuff and plays as a good model in studying crop plants (Khush, 2005). In rainfed paddy fields, water shortage has been well known as being a serious issue, especially in the seedling stage, during which plants are particularly sensitive (Hoekstra *et al.*, 2001). Pigmented rice cultivars are unique for organic farming to produce enriched antioxidant compounds in the grain for human health. However, it is lack of information on the defense response to water deficit and tolerant classification in pigmented rice genotypes. The aim of this investigation was a rapid water deficit tolerance in pigmented rice genotypes using multivariate parameters including proline accumulation, anthocyanin enrichment, MDA levels, photosynthetic pigment stabilization and growth reduction of mannitol-induced water deficit stress.

## Materials and Methods

**Plant materials and water deficit treatment:** Seeds of 8 pigmented rice (*O. sativa* L. spp. *indica*), [cv. Sangyod (SY), Khao Dang (KD), Kulab Dang (KLD), TD49, Klum Sakol Nakorn (KS), Klum Khonkaen1 (KK1), Klum Khonkaen2 (KK2) and Black sticky rice (BSR)] provided by Pathumthani and Sakolnakorn Rice Research Center were germinated on NB medium (Li *et al.*, 1993) and then cultured under ambient temperature at 25±2°C, 85-90% relative humidity. Photosynthetic photon flux (PPF) density was set at 60±5 μmol $m^{-2}$ $s^{-1}$ provided by fluorescence lamps with 16 h $d^{-1}$ photoperiod. Seven-days-old rice seedlings were transferred to NB-liquid medium under the photoautotrophic condition using vermiculite as supporting material for 14 days. Air-exchanges rate of culture vessel was normalized to 2.32 μmol $CO_2$ $h^{-1}$. Mannitol concentration in the culture medium was adjusted to 0 (control) or 100 mM mannitol (water deficit stress) for 4 days. Whole seedlings of rice were collected and keep at -80°C in the freezer prior to assay. Accumulation percentages of proline, anthocyanin and MDA were calculated according to water deficit stress/control (fold) and reduction percentages of photosynthetic pigments and growth characters were calculated as [1-(water deficit stress/control) ]× 100.

**Growth measurements:** Growth characteristics, shoot height (SH), root length (RL), fresh weight (FW) and dry weight (DW) of pigmented rice seedlings were evaluated.

**Photosynthetic pigments:** The chlorophyll a ($Chl_a$), chlorophyll b ($Chl_b$) and the total carotenoids ($C_{x+c}$) concentrations (mg $g^{-1}$ FW) were measured according to Lichtenthaler (1987) and Shabala *et al*., (1999) protocols.

**Lipid peroxidation:** Lipid peroxidation in rice seedlings were estimated as malondialdehyde (MDA) contents according to Hodges *et al*., (1999) method.

**Proline content:** Proline concentration was assayed according to Gilmour *et al*., (2000) protocol.

**Anthocyanin contents:** The total anthocyanin contents were extracted by acidic methanol and determined according to Reddy *et al*., (1995) and Harborne (1998).

**Isolation and analysis of genomic DNA:** Genomic DNA was isolated according to the protocol of Sambrook & Russell (2001) from 1 g fresh tissue and subjected to electrophoresis on 1.0% agarose gel. The intensity of genomic DNA was quantified by scanning densitometry using a gel document (SynGene) and the concentration of dsDNA was confirmed by measuring the absorbance at 260 nm in a spectrophotometer. The content of dsDNA was calculated using a Lambda DNA standard curve.

**Experimental design and cluster analysis:** The experiment was arraged as a completely randomized design (CRD) with five replications ($n$ = 5). Growth reduction, photosynthetic pigment degradation, proline accumulation, anthocyanin enrichment and MDA levels of rice seedlings grown under mannitol-induced water deficit were assessed in order to classify cultivars as either tolerant or sensitive using Ward's method of Hierarchical cluster analysis in SPSS software.

## Results

**Growth performances:** Shoot height (SH), root length (RL), fresh weight (FW) and dry weight (DW) of pigmented rice seedlings treated with 100 mM mannitol-induced water deficit for 4 days were decreased especially in SY, KD, KLD and TD49 (Table 1). In shoot height, the reduction percentage in mannitol-induced water deficit was ranged from 1.2% (KS cultivar) to 13.2% (KD cultivar). The reduction on root length showed a similar pattern as shoot height in range from 2.4% (KS cultivar) to 16.7% (SY cultivar). For the fresh weight, the reduction was varied from 3.5% (KK2 cultivar) to 21.6% (SY cultivar). Moreover, the dry mass reduction in pigmented rice seedlings was 2.1% (KK2 cultivar) to 18.3% (TD49 cultivar).

**Table 1. Reduction percentages of shoot height (SH), root length (RL), fresh weight (FW) and dry weight (DW) of pigmented rice seedlings treated with 100 mM mannitol compared to control condition for 4 days.**

| Genotypes | SH(%) | RL(%) | FW(%) | DW(%) |
|---|---|---|---|---|
| KS | 1.2 | 2.4 | 5.8 | 2.5 |
| BSR | 7.1 | 8.2 | 9.2 | 9.3 |
| KK1 | 4.4 | 4.8 | 8.1 | 3.8 |
| KK2 | 3.7 | 4.5 | 3.5 | 2.1 |
| SY | 9.1 | 16.7 | 21.6 | 10.4 |
| KD | 13.2 | 15.1 | 18.3 | 12.9 |
| KLD | 10.5 | 13.0 | 9.9 | 9.8 |
| TD49 | 12.7 | 12.7 | 16.9 | 18.3 |

**Photosynthetic pigments:** Chlorophyll a ($Chl_a$), chlorophyll b $Chl_b$, total carotenoids ($C_{x+c}$) and total photosynthetic pigment (TPP) concentrations in the leaf tissues of mannitol-induced water deficit for 4 days were dropped when compared to control (0 mM mannitol) treatment (Table 2). For the photosynthetic pigment degradation, those pigments were sharply degraded in SY, KD, KLD and TD49 (> 30% degradation) when seedlings were subjected to mannitol-induced water deficit. In contrast, the photosynthetic pigments in KS, BSR, KK1 and KK2 seedlings were stabilized (< 15% degradation) in water deficit stress (Table 2).

**Table 2. Degradation percentages of chlorophyll a ($Chl_a$), chlorophyll b ($Chl_b$), total carotenoids ($C_{x+c}$) and total photosynthetic pigment (TPP) contents of pigmented rice seedlings treated with 100 mM mannitol compared to control condition for 4 days.**

| Genotypes | $Chl_a$ (%) | $Chl_b$ (%) | $C_{x+c}$ (%) | TPP (%) |
|---|---|---|---|---|
| KS | 8.1 | 8.7 | 10.0 | 8.6 |
| BSR | 13.5 | 12.9 | 12.9 | 13.3 |
| KK1 | 10.6 | 10.8 | 12.4 | 11.0 |
| KK2 | 10.1 | 11.0 | 10.1 | 10.2 |
| SY | 33.5 | 35.2 | 30.6 | 33.1 |
| KD | 54.4 | 56.6 | 44.2 | 52.4 |
| KLD | 43.2 | 42.8 | 38.1 | 41.9 |
| TD49 | 37.8 | 36.0 | 48.3 | 39.9 |

**Proline, anthocyanin and lipid peroxidation levels:** Proline accumulation percentage was increased for 1.5 folds in KS, BSR, KK1 and KK2 when exposed to mannitol-induced water deficit. In contrast, proline accumulation in SY, KD, KLD and TD49 was lower than 1.5 folds (Fig. 1A). For anthocyanin accumulation, it was reached in only two rice cultivars, KS and KK2 when subjected to mannitol-induced water deficit (Fig. 1B). The increase MDA contents in SY, KD, KLD and TD49 were more than 1.5 folds, which were higher than those of KS, BSR, KK1, and KK2 (< 1.5 folds) (Fig. 1C). Thus, the lipid peroxidation in some pigmented rice seedlings was small amount, indicating as low rate of cell membrane damages.

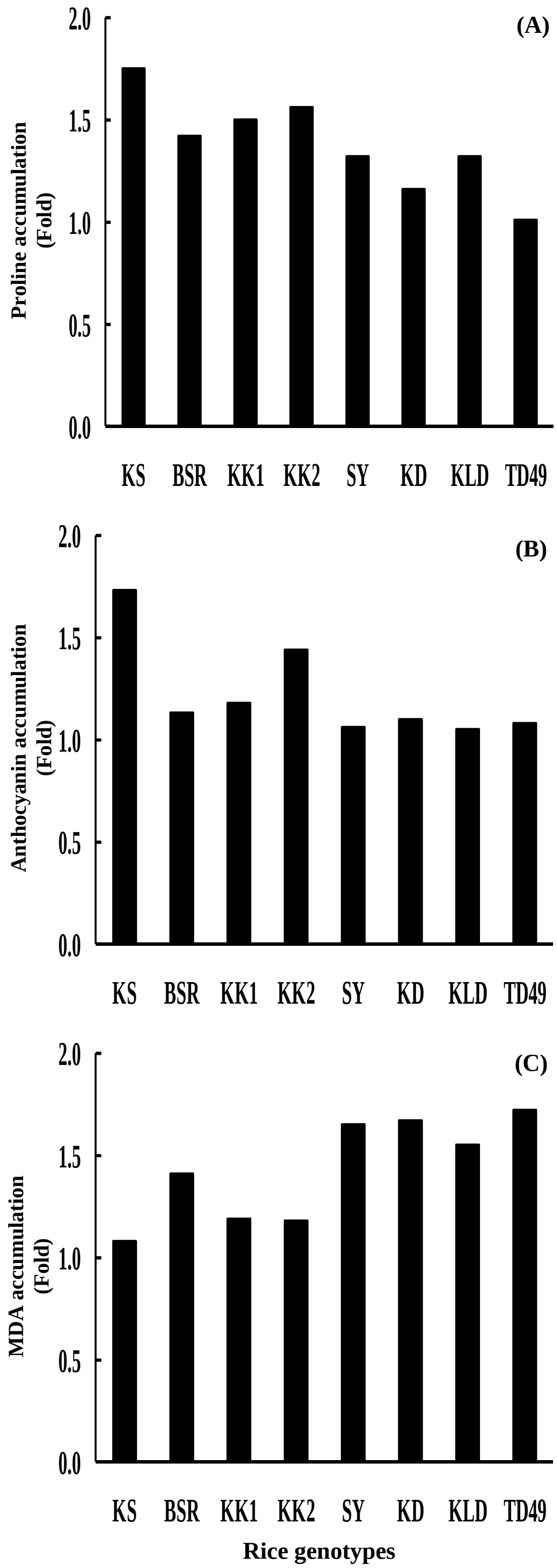


Fig. 1. Proline accumulation (A) anthocyanin accumulation (B) and lipid peroxidation accumulation percentages (C) of pigmented rice seedlings treated with 100 mM mannitol compared to control condition for 4 days.

**Biochemical and physiological relationships:** In present study, the positive correlation between the percentages of the total photosynthetic pigments stabilization and of the proline accumulation in drought stressed-seedlings as well as that of anthocyanin accumulation (Fig. 2). In the contrary, the negative correlation between the percentages of MDA content and of the proline or anthocyanin contents were demonstrated (Fig. 3). These relationships suggested that proline and anthocyanin contents inside the cell may function as photosynthetic pigments stabilization and as lipid peroxidation decrement including as the diminished deterioration effects on other compartments inside the cell.

**DNA contents:** A stability of genomic DNA has important effects on the controlling processes within cell, therefore, genomic DNA of selected seedling cultivars grown in drought stress were investigated. Stressed-seedlings of all tested cultivars showed reduction of genomic DNA contents when compared to theirs controls (Fig. 4A). The genomic DNA content of drought-tolerant cultivars were slightly decreased (12.06% in KS and 14.29% in KK2 stressed-seedlings), while those of drought-sensitive cultivars were highly decreased (46.93% in KD and 64.29% in SY stressed-seedlings) (Fig. 4B). These results showed that drought stress induced less DNA damages in drought-tolerant cultivars than in drought-sensitive cultivars of *indica* rice.

**Cluster ranking:** Growth reduction, photosynthetic pigment degradation, proline accumulation, anthocyanin enrichment and lipid peroxidation levels of rice seedlings grown under mannitol-induced water deficit were subjected to classify clusters of the group as water deficit tolerant, BSR, KS, KK1, KK2, and water deficit sensitive, KD, KLD, SY and TD49 using Ward's method (Fig. 5).

## Discussion

In the water deficit condition, pigmented rice seedlings showed the retardant in growth and photosynthetic pigments while the production of lipid peroxidation was increased. The increases of proline and anthocyanin accumulations against water stress were also detected in all rice genotypes. Furthermore, the correlation of the proline and anthocyanin accumulations and reduce cellular damages (photosynthetic pigment stabilization and the MDA content) under water deficit stress was demonstrated. Water deficit stress can cause seedlings injury by various mechanisms related to osmotic and oxidative damages at cellular levels. In water deficit stressed plants, various kinds of stress-inducible genes are differently expressed (Seki *et al.*, 2002). The gene expressions depend on the genetic content inside each of the rice cultivars and affect on stress tolerant efficiency of whole seedling. Under the water deficit stress, chloroplast ultrastructures are the first targets to be damaged in the cellular levels since it is the major site of ROS production (Munné-Bosch & Peñuelas, 2003). An enriched ROS in stressed tissues impairs cellular membrane and organelles which affects on the integrity of cell.

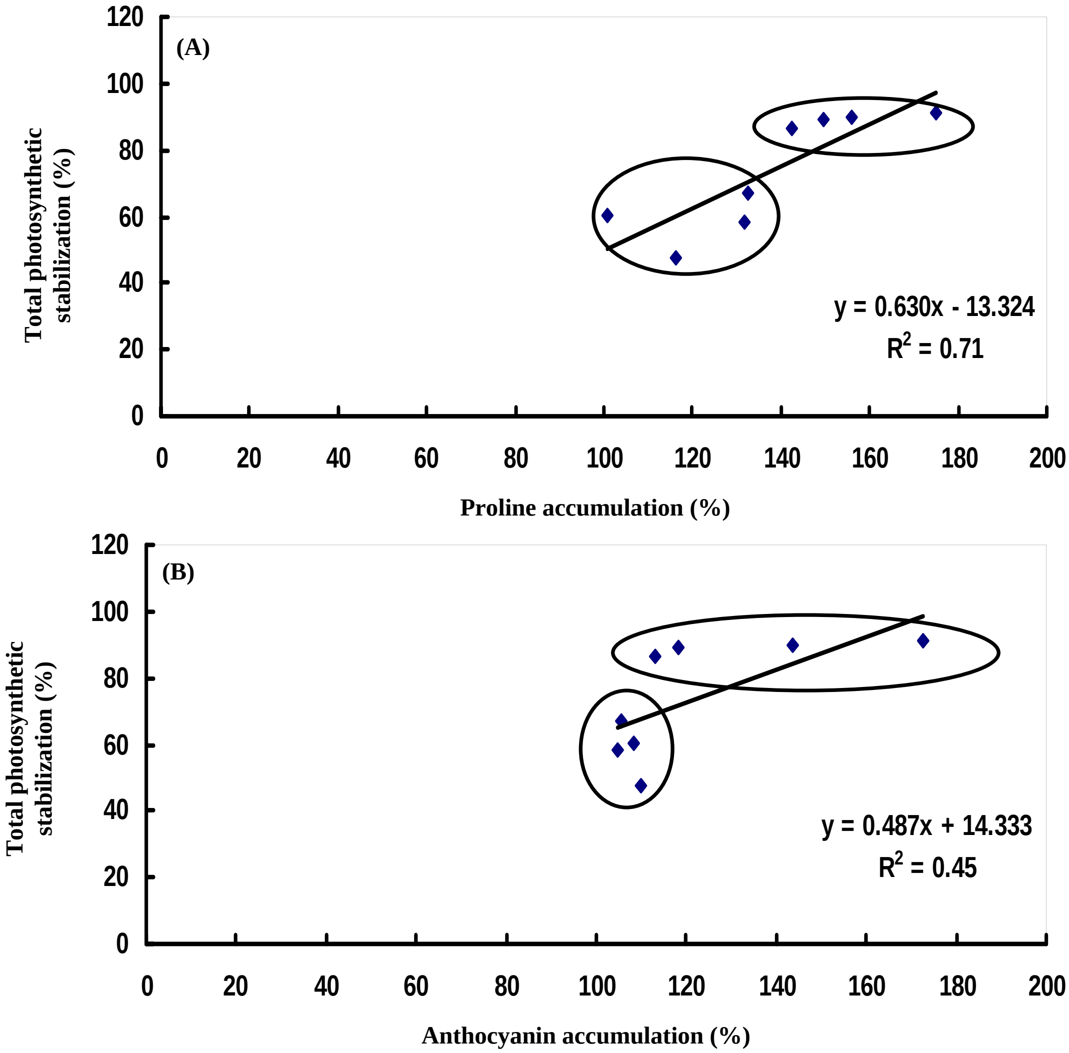


Fig. 2 Correlation between the percentage of total photosynthetic stabilization and the percentage of proline accumulation (A), total photosynthetic stabilization and anthocyanin accumulation (B) in pigmented rice seedlings treated with 100 mM mannitol for 4 days.

MDA, a product of lipid peroxidation, refers to oxidative degradation of lipid in cell membrane. The oxidative stress in the plant cells has been identified by high level of MDA in rice seedlings subjected to drought stress. Some rice cultivars accumulate less MDA than other cultivars under mannitol-induced water deficit. These data imply a less water stress-induced oxidative injury in some rice cultivars as shown by the stability of photosynthetic pigments in present results (Table 2). This evidence implies photosynthetic capacity resulting in the decreased accumulation of seedling growth performances. The increased proline accumulation in stressed-plants may be a principle adaptation to defeat the stressed conditions. In our experiments, proline contents in all cultivars of pigmented rice seedlings treated with water deficit were enriched similar to those of sorghum (Yadav *et al.*, 2005), bell pepper (Nath *et al.*, 2005), maize (Efeoğlu *et al.*, 2009), barley (Thameur *et al.*, 2011) and wheat (Hong-Bo *et al.*, 2006). Proline accumulation in stressed plants has been well established to play a key role as osmoregulation defense mechanism, leading to prevent the cell osmotic pressure and survive in the extreme conditions (Sankar *et al.*, 2007). In fact, proline accumulation in stressed plants may function as an osmoprotectant compound and reserve energy (Al-Khayri & Al-Bahrany, 2004). Moreover, high proline accumulations in plant cells may reflect the reduction in lipid peroxidation products, acting as free radical scavenger (Hsu *et al.*, 2003). Proline composes of C=C or C=O double bonds within the structure, which plays as a central core to protect plants against singlet oxygen and free-radical induced damages (Matysik *et al.*, 2002). Moreover, proline binding with redox-active metal ions can prevent the biological tissues, damaging by $OH^{\bullet}$ free radicals (Hsu *et al.*, 2003).

Anthocyanin biosynthesis in plant cells regulated by several environmental conditions including osmotic stress has been well-known (Pourcel *et al.*, 2006; Chutipaijit *et al.*, 2009). In the resurrection plants, an increase in anthocyanin contents was observed during dehydration for 3-4 folds (Sherwin & Farrant, 1998). Moreover, earlier investigation was observed the increase in anthocyanin accumulation under drought stress in maize (Doge, Luce and Vero cultivars) (Efeoğlu *et al.*, 2009). In addition, an enhanced resistance to water loss by enriched anthocyanin in plant cells has been reported. Osmotic potential of the cells might modify by the glycosides attached to the anthocyanin in vacuoles, causing to minimizing the water loss through transpiration system (Srivalli *et al.*, 2003). Moreover, anthocyanins belonging to flavonoids family may also contribute as antioxidant to protect the scavenging of ROS (Dorman *et al.*, 2003). Anthocyanin can directly scavenge $H_2O_2$ and $O_2^-$ (Noda *et al.*, 2000). They are also likely to act as reducing agents for general peroxidase which are extremely abundant in the cytoplasm and to inhibit the formation of $OH^•$ radicals through Haber-Weiss-Fenton reactions by chelating transition metals (Yamasaki & Grace, 1998; Batool *et al.*, 2010).

For cellular stresses, DNA is particularly sensitive to $OH^•$ radical which can induce various damages, i.e. breakage of DNA strand and base hydroxylation. Therefore these may alter genetics inside the cell by mutations or rearrangements (Rahman *et al.*, 2000; Sundaravalli *et al.*, 2005). Previous reports showed that anthocyanin could make complexes with other molecules such as DNA (Mas *et al.*, 2000; Kong *et al.*, 2003). Formation of the anthocyanin-DNA complex may help to maintain the structural integrity of DNA when it is exposed to abiotic stress. Therefore, an increase anthocyanin in rice cultivars may protect the DNA damages when exposed to water deficit stress. In case of antioxidants, anthocyanin is a small molecule, which may effectively function against water deficit when compared to proline or other compounds; therefore there are several reports in many plant species, which identified as water deficit tolerance without high level of proline contents (Ashraf & Foolad, 2007).

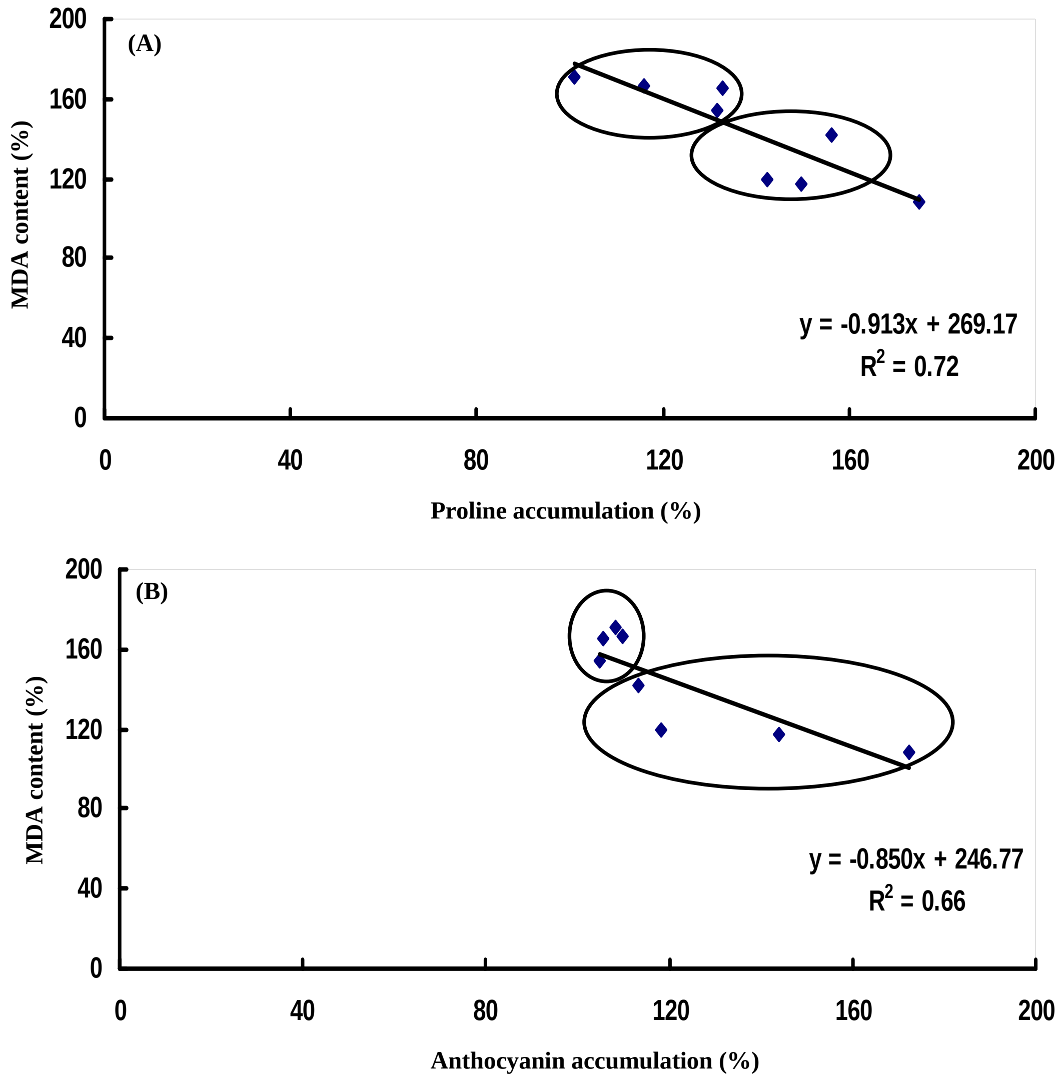


Fig. 3 Correlation between the percentage of MDA content and the percentage of proline accumulation (A), MDA level and anthocyanin accumulation (B) in pigmented rice seedlings treated with 100 mM mannitol for 4 days.

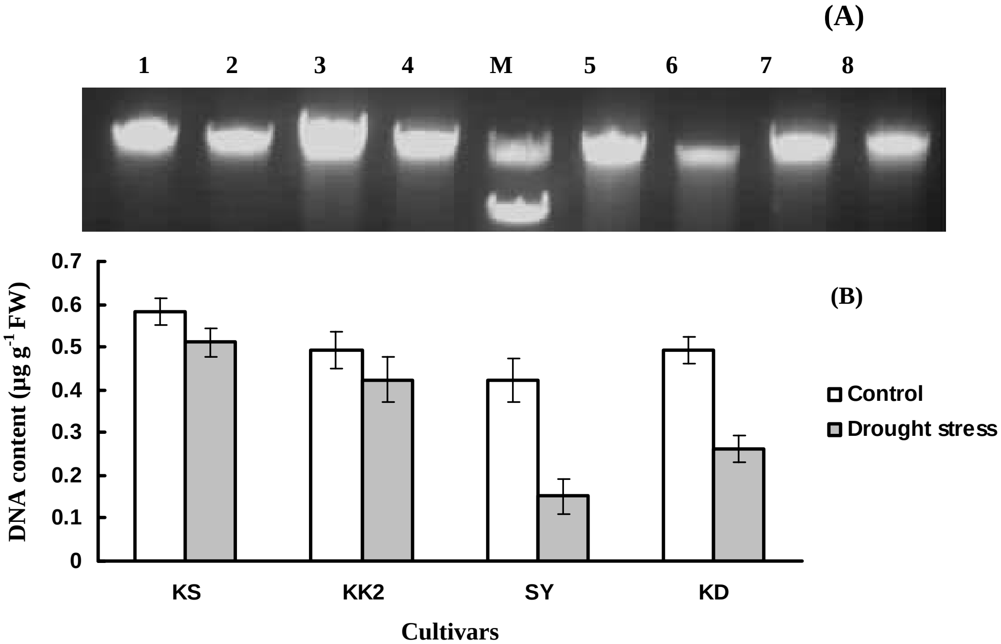


Fig. 4. DNA content in pigmented stressed-seedlings of rice genotypes subjected to 100 mM mannitol under photoautotrophic system. M, DNA marker (λ/*Pst*I); Lanes 1, 3, 5 and 7 are unstressed-DNA of KS, KK2, SY and KD, respectively; Lanes 2, 4, 6 and 8 are stressed-DNA of KS, KK2, SY and KD, respectively (A). Quantification of genomic DNA in pigmented rice seedlings treated with 100 mM mannitol or without mannitol for 4 days. Error bars represent by ± SE (B).

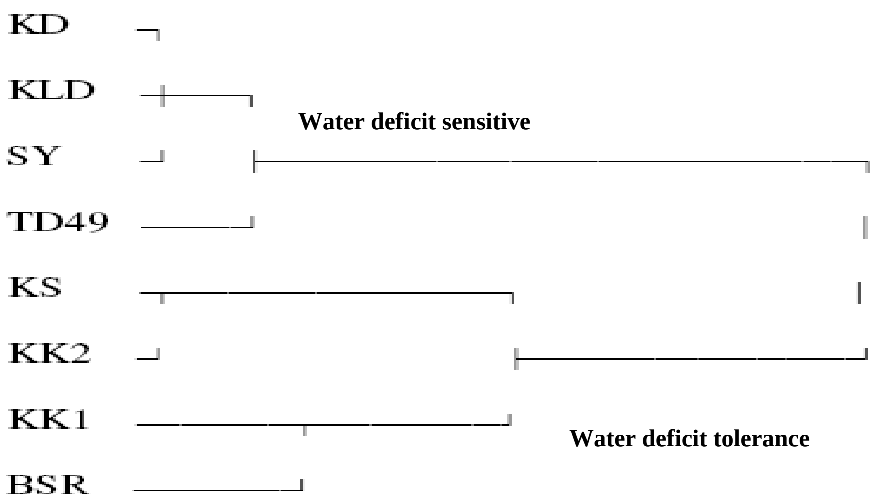


Fig. 5 Ward's dendrogram for pigmented rice genotypes to classify as water-deficit sensitive, KD, KLD, SY, TD49 and water-deficit tolerant, KS, KK2, KK1 and BSR using growth reduction , photosynthetic pigment degradation, proline accumulation, anthocyanin enrichment and MDA levels

## Conclusion

The biochemical (proline, anthocyanin and MDA levels) and physiological characters (photosynthetic pigments and growth characters) of pigmented rice seedlings under mannitol-induced water deficit stress were significantly changed. The level of anthocyanin and proline accumulations in pigmented rice seedlings was positively related to pigment stabilization and prevent the toxic damage from ROS with low level of MDA expression. Growth performances, photosynthetic pigments, proline, anthocyanin accumulation and MDA levels in rice seedlings grown under mannitol-induced water deficit were subjected to classify into water deficit tolerant, BSR, KK1, KK2, KS and water deficit sensitive cultivars, KD, KLD, SY, TD49.

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