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PHYSIOLOGICAL RESPONSE AND ANTIOXIDANT ACTIVITIES OF RICE AND GRASS WEEDS TO FLOODING DURING GERMINATION

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ABSTRACT

Weeds are among the most important biological constraints to successful production of direct-seeded rice (DSR) and therefore, herbicides have become major method of weed control. Early flooding is an effective tool to manage weeds, but flooding negatively effects on the seedling establishment of rice. Three rice genotypes, Khao Hlan On (KHO), Mazhan Red (MR) and, IR64, and three weeds, *Echinochloa colona*, *Echinochloa crus-galli*, and weedy rice were evaluated to understand the physiological and anioxidative mechanisms under flooding. Dry seeds were sown at 1-cm soil depth and flooded to 0, 1, 5 and 10 cm depths. Malondialdehyde (MDA), total phenolic content (TPC), antioxidative enzymes and amylase activity were assayed in germinated seeds at 4 days after sowing (DAS). Seedling emergence and growth were assessed at 21 DAS. KHO and MR showed a higher seedling emergence and growth under flooding. Lower MDA content, higher TPC, higher superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (POX) and higher amylases activities of KHO and MR were associated with their high seedling emergence under flooding. In contrast, higher MDA, lower TPC, lower antioxidative enzymes and amylase activity of IR64 rice genotype, were associated with lower emergence and growth. High MDA, low TPC, lower antioxidative enzymes and amylase activity of two *Echinochloa* species confirmed the susceptibility to early flooding. Weedy rice had low seedling emergence and poor growth, the lowest MDA, the highest TPC, high antioxidant activity and lower amylase activity. Poor seedling emergence and growth of *Echinochloa* species and weedy rice under flooding indicate that flooding to 5 cm and 10 cm is an effective tool in managing these weeds in wet seeded rice when flood-tolerant rice genotypes are used.

Key words: Antioxidative enzymes, Amylase activity, *Echinochloa* spp., Flooding, Lipid peroxidation, Total phenolic content, Weedy rice.

INTRODUCTION

The widespread introduction of direct seeding rice cultivation has led to aggravate the weed problem and increase in reliance on herbicides. Of several weeds affecting rice production, weedy rice is considered as the most dominant weed followed by *Echinochloa* species (Rao *et al.,* 2007). Weedy rice is taxonomically classified as the same species as cultivated rice (*O. sativa*), but is strongly characterized by its seed shattering and dormancy, which apparently increased the distribution of this species (Cao et al., 2007). In Asia, weedy rice has become a serious threat to the sustainability of rice production in many rice producing countries, including in the Philippines, Vietnam, Malaysia, India, Sri Lanka and Thailand. Occurrence of weedy rice has increased due to extensive adoption of direct-seeded rice (DSR) (Pyon *et al.*, 2000 and Watanabe *et al.*, 2000). In Sri Lanka weedy rice exhibit more morphological diversity and has become enormous challenge to farmers and scientists in Asia region (Marambe, 2009). In the Philippines, weedy rice infestation in rice fields has increased to 48% (Baltazar and Janiya, 2000). In Vietnam, weedy rice causes an average yield loss of 18% in direct-seeded rice (Chin, 1997). In Malaysia, RM 90 million estimated yield loss of rice was caused by the weedy rice infestation (Azim and Rezaul *et al.*, 2008). Management of weedy rice is difficult because of absence of selective herbicides to be used within the rice crop and its morphological similarity to the cultivated rice.

Echinochloa species are one of the major weeds in DSR. Among the *Echinochloa* species; *Echinochloa crus-galli* (L) Beauv. and *Echinochloa colona* (L) are found to be the major troublesome weeds in rice fields in many rice growing countries. *Echinochloa crus-galli* is ranked as the world's third worst weed, and it is one of the most problematic weeds in rice (Holm et al., 1977). *Echinochloa crus-galli* has been reported to reduce rice yields by 38 % to 64 % depending on the rice cultivar (Stauber *et al.*, 1991). *Echinochloa crus-galli* is particularly abundant in flooded rice fields where they reduce yield by 40 % (Smith *et al.,* 1977). Higher adaptation of direct seeding aggravated the problem of *Echinochloa* species, hence increased the dependency on herbicides.

Water management is the key component of integrated weed management in DSR (Rao *et al.*, 2007). Flooding to 5-7 cm prevented the growth of major weeds in rice and 10 cm flooding had prevented germination of most weed seeds in rice fields (Williams, 1987). Flooding delayed emergence and growth of the weeds in rice fields (Kent and Johnson 2001). *Echinochloa crus-galli* can emerge and survive under the flooded condition but not the *E. colona* (Sparacino *et al.*, 2002). *Echinochloa colona* can be suppressed using a shallow water depth (Kim and Moody 1989). Flooding to a depth of 5 cm or more was able to suppress germination and emergence of weedy rice in Vietnam (Chin, 2001). Flooding to 5-10 cm inhibited the emergence of weedy rice in Malaysia (Azim and Karim 2008). Flooding depth of 5 -10 cm at seeding time is sufficient to suppress weedy rice in water seeding (Chin *et al.*, 2000).

Flooding leads to oxidative stress through an increase in reactive oxygen species (ROS), such as superoxide ($O2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) (Blokhina *et al.*, 2003). Elevated ROS damage to membrane integrity, changes the lipid composition and induction of lipid peroxidation (Blokhina *et al.*, 2003). Malondialdehyde (MDA) content is an end product derived from the breakdown of polyunsaturated fatty acid and is widely used as an indicator of lipid peroxidation. Plants have reactive oxygen-scavenging systems consisting of several enzymatic and non-enzymatic antioxidants, which can neutralize the ROS. Among the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) play key roles in ROS scavenging in cells (Damanik *et al.*, 2010). The SOD provides the first line of defence against the toxic effects of elevated levels of ROS by converting superoxide ($O2^-$) to hydrogen peroxide (H_2O_2). The CAT directly converts H_2O_2 to water and oxygen. Which, APX is involved in scavenging of H_2O_2 into water- water and ascorbate-glutathione cycles and utilizes ascorbate as an electron donor.

Tolerance of anaerobic conditions at early stages is a prerequisite for effective direct-seeded rice establishment under flooding conditions. Tolerant rice genotypes germinate faster and their coleoptiles grow at a relatively faster rate to emerge from flooded soil (Ismail *et al.*, 2009). Understanding the response mechanisms of weeds and rice to early flooding could be helpful in the development of integrated approaches for weed control and in characterizing physiological and biochemical traits associated with flood tolerance to develop flood-tolerant rice. Therefore this study aimed to determine the physiological traits associated with tolerance of flooding during germination and seedling growth of of *Echinochloa* spp, *Oryza sativa* f. *spontanea* (weedy rice type) and cultivated rice genotypes. Information on mechanisms of physiological responses to flooding could be used in developing flood tolerant rice genotypes.

MATERIALS AND METHODS

Greenhouse experiments were conducted at the International Rice Research Institute (IRRI), Los Baños, Philippines, with three cultivated rice genotypes, Khao Hlan On (KHO), Mazhan-red, (MR) and IR64, two *Echinochloa* species (*E. crus-galli* and *E. colona)* and weedy rice ecotype from the Philippines. Treatments consisted of four levels of flooding depths (0, 1, 5 and 10 cm), and 0 cm (saturated soil) was maintained as the control. Plastic pots (10 cm x 10 cm x 6 cm) were filled with sterilized soil (clay 37 %; sand 15 %; silt 48 %; pH 6.11 and organic carbon 0.95 %). Forty five dry seeds of each rice genotype and weedy rice and Two hundred seeds of both *E. crus-galli*, *E. colona* were sown at 1 cm soil depth and flooded at four depths (0, 1, 5, and 10 cm). he different flooding levels were continuously maintained until 21 days after sowing (DAS) in the flooding chamber. The seeds were allowed to germinate and grow for 21 days. The studies were conducted using a split-plot arrangement within a randomized complete block design with three replicates, where, flooding depths were maintained as main plot and weeds and rice varieties as sub plots. Lipid peroxidation (MDA content), total phenolic content, antioxidative enzyme (SOD, CAT, APX and POX) and amylase activity were determined in germinated seeds at 4 DAS. At 7, 14, and 21 days after seeding, the seedlings were harvested and shoots and roots length were measured. The numbers of emerged seedlings were recorded at 21 DAS.

Determination of lipid peroxidation

Lipid peroxidation was determined by measuring Malondialdehyde (MDA) formation using the thiobarbuturic acid method described by Hodges *et al.* (1999). Malondialdehyde equivalents were calculated using equations given in Hodges *et al.*, (1999).

Determination of total phenolic content

Crude extraction of phenolics was done following the method of Xu and Chang (2008) with a slight modification. Total phenolic content was determined by spectrophotometric method using Folin-Ciocalteu's phenol regent (Makkar *et al.*, 1993). A calibration curve was prepared using standard gallic acid solution (Sigma Aldrich, Singapore) and results were expressed as gallic acid equivalents.

Enzyme extraction and dialysis

A pre-weighed fresh seed sample (0.1 g) was frozen and ground to fine powder with liquid nitrogen and extracted with 2 ml of iced cold extraction (1Mm PMSF, 1 Mm DTT, 0.01 % Triton x 100, 1Mm PEG 4000, 0.5 Mm HEPES pH 7.5, 0.1 mM EDTA). The extraction was centrifuged at 20,000 x g and 4 ^{0}C for 20 minutes. One ml of the fresh supernatant was dialyzed against Viskase Membra- Cel (MWCP 7000, USA) over night with 0.005 Mm HEPES (pH 7.8) and the dialyzed extract was used for the SOD, CAT, APX and POX assays.

Superoxide dismutase (SOD) assay

SOD activity was measured following Asada *et al.* (1973). One unit of SOD activity was defined as 50% inhibition of the control rate. SOD activity was calculated following formula; Assay SOD units = (Vc/ Vs) -1; Where Vc is the reaction rate of the control and Vs is the reaction rate of the sample.

Catalase (CAT) assay

CAT activity was measured following Beers and Sizer (1952). The expression of one unit of CAT activity is nmol H_2O_2 decomposed per minute, calculated using its extinction coefficient of 39.4/M/cm.

Ascorbate peroxidase (APX) assay

APX activity was measured following the method of Nakano and Asada (1987). The expression of one unit of APX activity is nmol ascorbic acid oxidized per minute, calculating using its extinction coefficient of 208 m/M/cm.

Guaiacol peroxidase (POX) assay

POX activity was measured following the method of Zhang and Kirkham (1994) with slight modification. The expression of one unit of POX activity is nmol H_2O_2 decomposed per minute, calculated using its extinction coefficient of 26.6 mM^{-1} cm^{-1}.

Amylase activity assay

Total amylase and α-amylase activity were measured using the method of Bernfeld (1955). The total protein concentration was determined using the Bradford method (Bradford 1976), with brovine serum albumin (BSA) as a protein standard. One unit of amylase activity is defined as µmoles maltose produced per minute and activity was expressed in units per milligrams protein.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using STAR for Windows version 2.1(IRRI, 2014). Differences among treatments were compared using least significant differences (LSD) at P=0.05. Relationships between different attributes were determined using simple linear regression.

RESULTS AND DISCUSSION

Seedling emergence of rice genotypes was affected by flooding. Among the rice genotypes; emergence was highest in KHO and MR under each flooding depth (Figure 1). Emergence of IR64 was reduced by 55 % with 5 cm flooding and by 64 % with 10cm. Flooding was less marked with KHO and MR than the IR64. Emergence of *E. colona* was completely suppressed by the flooding treatments (1, 5, and 10 cm) whereas; *E.crus-galli* emergence was reduced by 62 % with 10 cm flooding. Agreed to these results, Kim and Moody (1989) found that, *E. colona* can be suppressed using a shallow water depth.

Emergence of weedy rice was reduced by 64 % with 1 cm flooding, by 84 % with 5 cm flooding and by 94 % with 10 cm flooding. Chin, (2001) reported that, flooding depth of 5 cm or more was able to suppress germination and emergence of weedy rice in Vietnam.

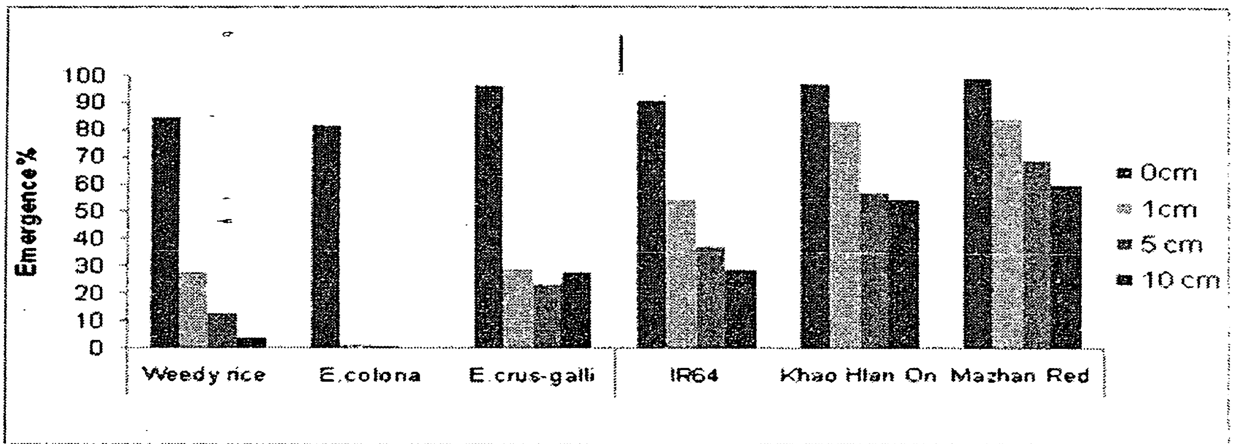

Figure 1. Emergence of weedy rice, *E. colona and E. crus-galli* and three rice genotypes grown in increasing flooding depths at 21 DAS.

Note: Vertical bar represents the $LSD_{.05}$.

Among the rice genotypes, greater reduction of shoot length was observed in IR64 under flooding, but reduction was lower in KHO and MR rice genotypes (Figure. 2). More than 50 % shoot length reduction in IR64 was observed under 10 cm flooding compared to control condition. Weeds showed highly reduced shoot length under flooded conditions than the rice genotypes. Shoot length of *E. crus-galli* was reduced by 50 % with 1 cm flooding and 78% with 10 cm flooding. Shoot length of weedy rice was reduced by an average 70 % with 5 and 10 cm flooding.

Increasing flooding depth decreased the root length in all rice genotypes and weeds (Figure 3). Among the rice genotypes, IR64 showed highly reduced root length under the 5 and 10 cm flooding, while KHO and MR showed only 30 % reduction of root length. Greater reduction of root length was observed in weeds than the rice under the flooding condition. Forty two and 85 % reduction of root length was observed in *E.crus-galli* when flooded to 5 and 10 cm. Root length of weedy rice reduced by 61 % with 1 cm, by 73 % with 5 cm and by 88 % with 10 cm flooding than the control.

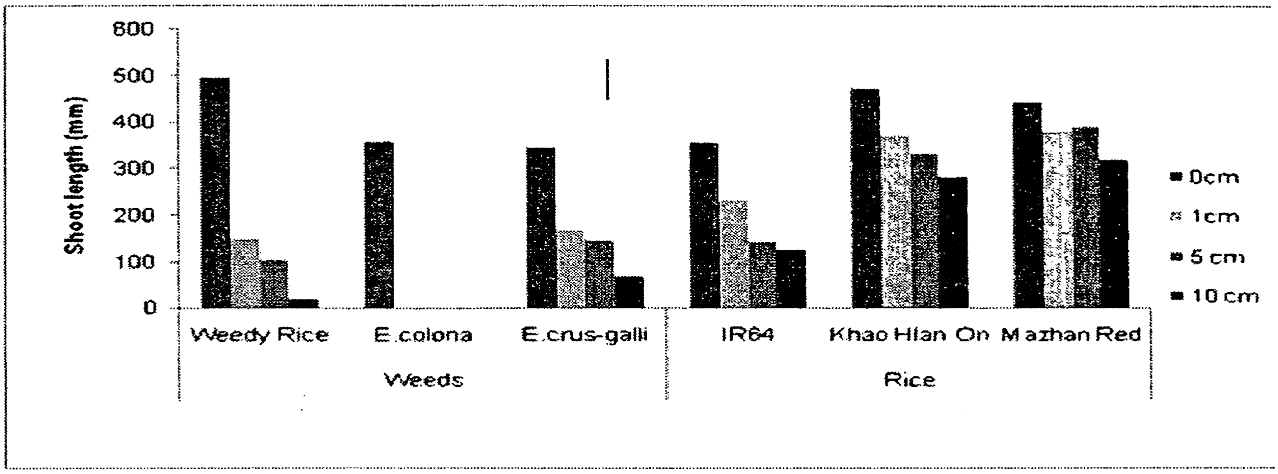

Figure 2. Shoot length variation of weedy rice, *E. colona and E. crus-galli* and three rice genotypes grown in increasing flooding depths at 21 DAS.
Note: Vertical bar represents the LSD$_{.05}$.

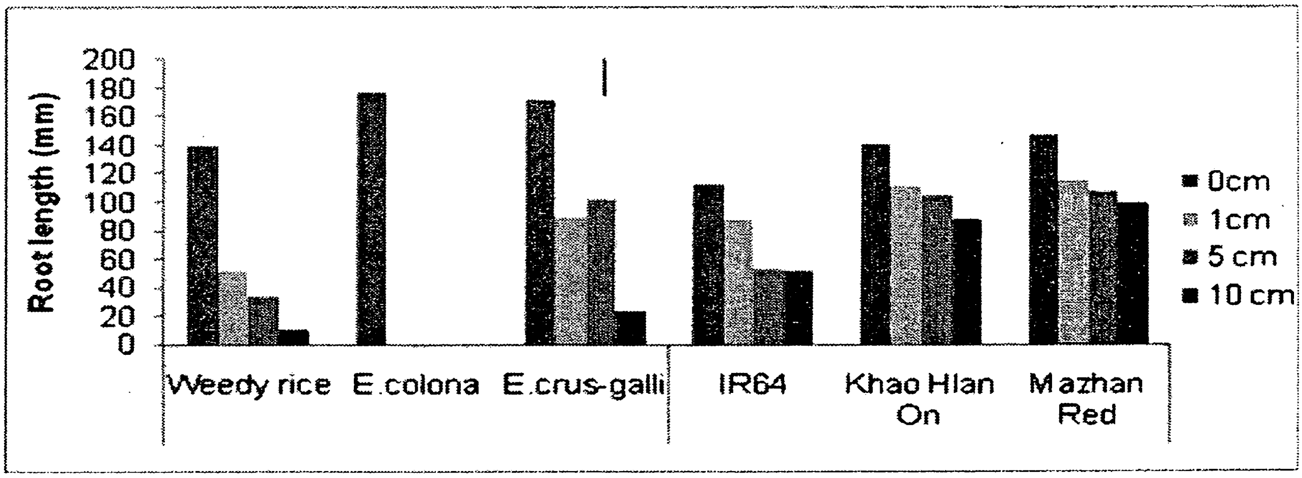

Figure 3. Root length variation of weedy rice, *E. colona and E. crus-galli* and three rice genotypes grown in flooding depths at 21 DAS.
Note: Vertical bar represents the LSD$_{.05}$.

Lipid peroxidation in germinating seeds

Among the rice genotypes, KHO and MR exhibited lower MDA content, while higher MDA content was observe in IR64 and there was no significant effect of flooding on the MDA content of KHO and MR (Figure 4). Lower level of MDA content exhibited in submergence tolerance rice genotypes under complete submergence condition (Ella *et al.*, 2003). The strong negative correlation was observed between the emergence of rice genotypes and MDA content under different flooding depths as shown in Fig 5. Lower emergence percentage IR64 rice genotype may be due to higher MDA content under flooding conditions. These results revealed that, KHO and MR can withstand flooding stress and, therefore, reduced content of MDA is an important indicator for the tolerant ability of rice genotype to flooding during germination.

Weed species responded differently to the flooding during germination. MDA content increased with increasing flooding depth in *E. colona* than *E. crus-galli*. Lower MDA content of *E. crus-galli* may be associated with the high emergence under flooding than *E. colona*. *E. crus-galli* can germinate and grow for a long period of time in a completely oxygen free environment (Mary *et al.*, 1981). In contrast, weedy rice showed the lowest MDA content under flooding.

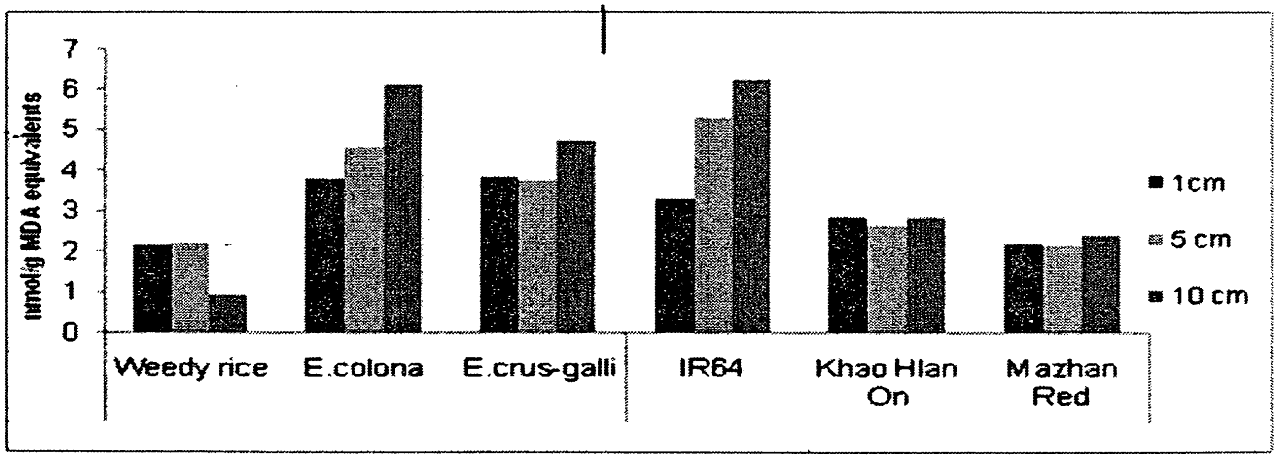

Figure 4. Malondialdehyde (MDA) content at 4 DAS of weedy rice, *E. colona*, *E. crus-galli* and three rice genotypes with different flooding depth.

Note: Vertical bars represent the $LSD_{.05}$.

Total phenolic content in germinating seeds

Weedy rice exhibited highest phenolic content (Fig. 6). MR had highest phenolic content among the rice genotypes. Meanwhile, weedy rice had highest phenolic content and lowest MDA content. This agrees with the report of Muntana *et al.* (2010) that, MDA content in red rice is relatively low compared to their white rice due to increased level of phenolic content. Similarly, Oki et al., (2002) reported that, pigmented rice, such as red and black rice, composed of high content of phenolic compounds than white rice. *E. crus-galli* had higher phenoic content than the *E. colona*. Meanwhile, *E. crus-galli* exhibited a lower level of MDA and, this might be related to the flood tolerant ability of *E. crus-galli* than *E. colona*.

Antioxidant enzymes activity

Increased SOD activity was observed in KHO, MR and weedy rice under flooded condition than in the control (Figure 7). IR64 had lower SOD activity under flooding than control. This may be probably due to higher activation of SOD activity in KHO and MR than IR64 under flooding condition. This may be the reason to have higher MDA content in IR64, because of exhibited SOD activity may not be enough to scavenge the free radicals in IR64 under flooding. This results further supported by the strong negative correlation observed between MDA content and the SOD activity (r =-0.79 ***) as shown in Table 1. *E. crus-galli* exhibited higher SOD activity compared to the *E.colona* with 10 cm flooding.

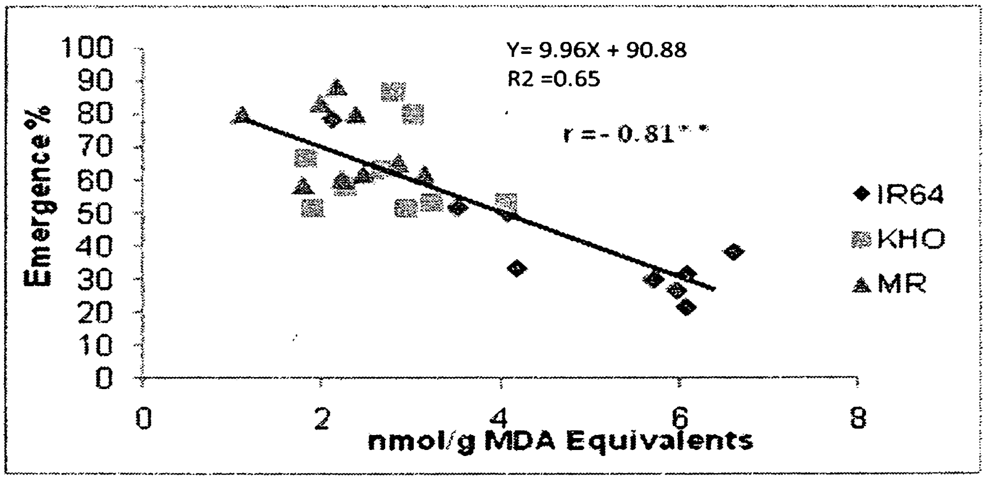

Figure 5. Relationship between emergence and MDA content of three rice genotypes at different flooding depths at 4 DAS.

Note: ** Correlation significant at P< 0.01 level.

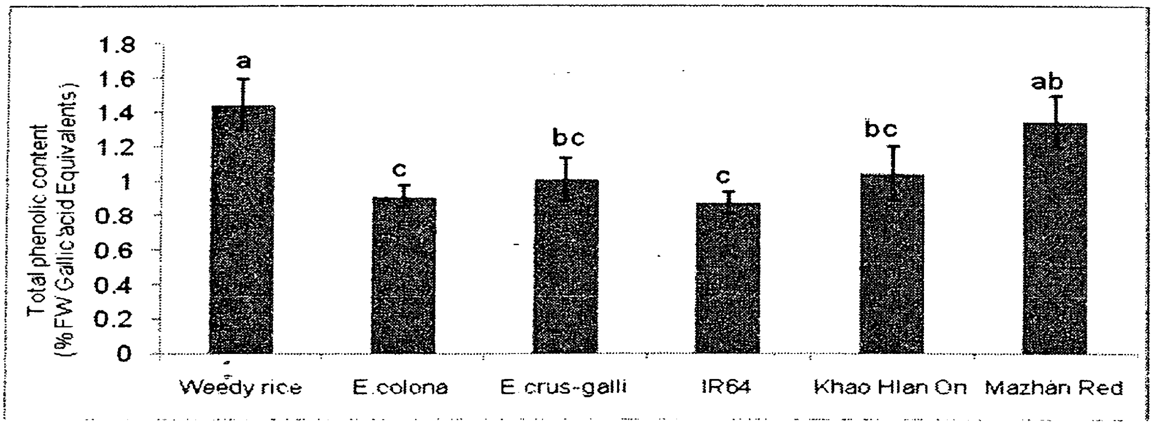

Figure 6. Total phenolic content of weedy rice, *E. colona*, *E. crus-galli* and three rice genotypes, across the flooding depths at 4 DAS. Vertical bars represent the ±SE of the means.

Note: Different letters indicate significant differences at ($P < 0.05$) according to LSD.

The CAT activity of rice genotypes increased under flooding than the control (Figure.8). KHO had higher CAT activity than the IR64 under flooding. This is further supported by the strong negative correlation (-0.6**) between MDA and CAT activity as shown in Table 1. This implies that KHO is having the steady state between SOD and CAT activation under flooding stress to alleviate plants from oxidative stress damage. Matés (2000) stated that, the combined action of SOD and CAT is critical in mitigating the effect of oxidative stress. Das et al., (2004) found that, rice seedling subjected to submergence increased the CAT activity of submerge tolerant rice cultivar than the intolerant rice cultivar.

The APX is also considered to be another major H_2O_2 scavenging enzyme in plants related to stress. KHO and MR exhibited increased APX activity with flooding than in control (Figure. 9) whereas IR64 showed reduced levels of APX activity. Further, there was a negative correlation (-0.47*) between APX activity and MDA content as shown in Table 1. This relationship also explained the scavenging activity of APX against to oxidative stress under flooding.

Flooding resulted in decreased POX activity of KHO, MR, IR64, weedy rice and *E. crus-galli*, except *E. colona* at 4 DAS (Fig. 10). POX also considered as an important antioxidant in plant system reacts against to stress conditions. Results showed a, reduced level of POX in rice genotypes under flooding than the control. KHO and MR exhibited higher POX activity compared to the IR64 under flooding. These results also further support for the higher ROS scavenging of KHO and MR than the IR64 during germination under flooding. POX increased in Cd-exposed plants of *T. aestivum* (Milone *et al.*, 2003). A concomitant increase in POX activity in both the leaf and root tissues of *Vigna radiate* (Panda, 2001), *O. sativa* (Koji, 2009) has also been reported under salinity stress.

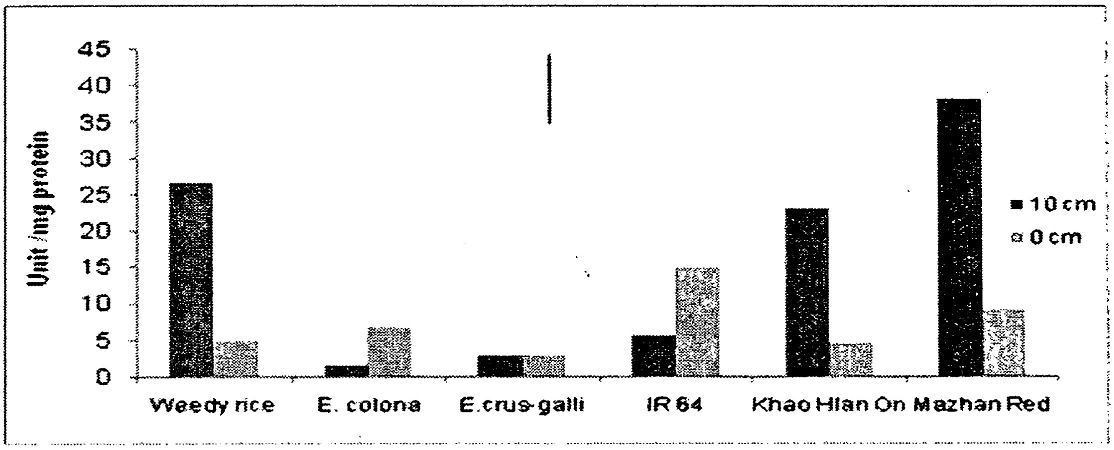

Figure 7. SOD activity of weedy rice, E. colona, E. crus-galli and three rice genotypes as affected by different flooding depth at 4 DAS.

Note: Vertical bar represents the $LSD_{.05}$.

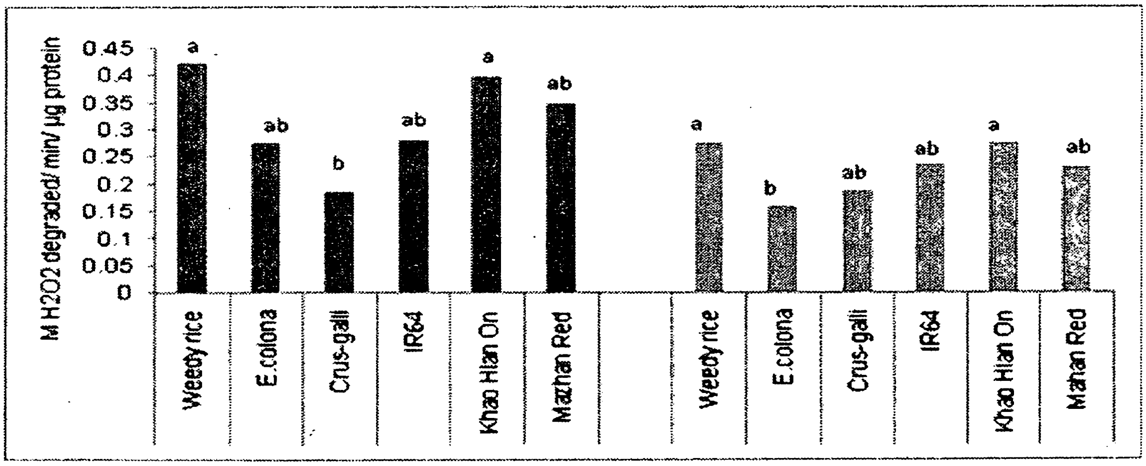

Figure 8. CAT activity of weedy rice, E. colona, E. crus-galli and three rice genotypes with flooding depths at 4 DAS.

Note: Different letters indicate significant differences at ($P< 0.05$) according to HSD

Amylase activity

KHO and MR had higher amylase activity than IR64 under flooding. Reduction of total amylase activity with flooding in KHO and MR was twofold, whereas IR64 showed 17 fold reductions. The ability to degrade starch into soluble sugars under flooding stress plays a key role in the ability of seedlings to survive and grow faster under flooded condition (Ismail *et al.*, 2009).

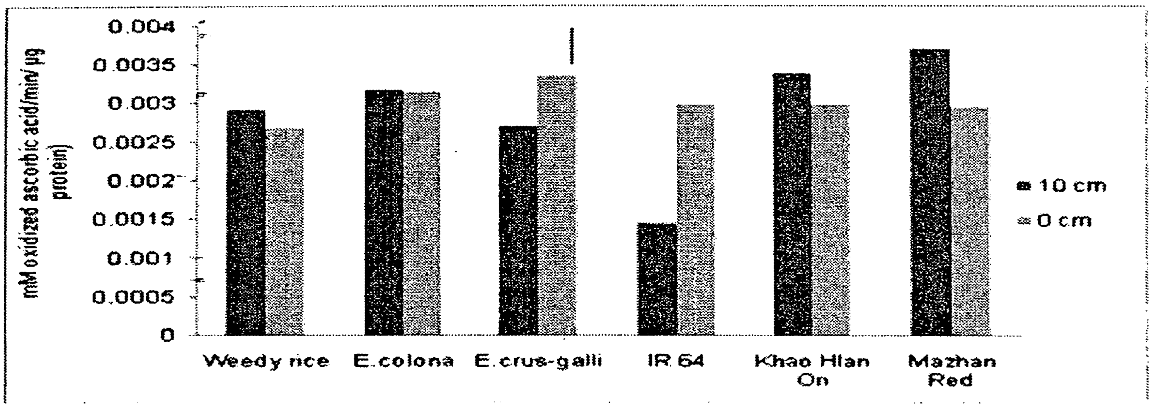

Figure 9. APX activity of weedy rice, *E. colona, E. crus-galli* and three rice genotypes with different flooding depth at 4 DAS.

Note: Vertical bar represents the $LSD_{.05}$.

During cereal seed germination, α amylase in the aleurone layer plays an important role in hydrolyzing the endosperm starch into metabolizable sugars, which provide energy for the growth of roots and shoots (Akazawa and Hara-Nishimura, 1985). In flooded soil, *E. crus-galli* had higher total amylase activity than the *E. colona*. Reduction of amylase activity of *E. crus-galli* and *E. colona* was three and four fold respectively under flooding than the control. These results indicate that, *E. crus-galli* has higher ability to germinate under flooding condition compared to *E. colona*. The results further suggest that, genotypes with higher amylase activity are able to emerge and grow faster under flooding. Weedy rice had eight fold reduction of total amylase activity under flooding condition compared to the control. Further, weedy rice showed reduced α and β amylases compared to KHO and MR under flooding. These results explain why weedy rice is not able to germinate under flooding condition.

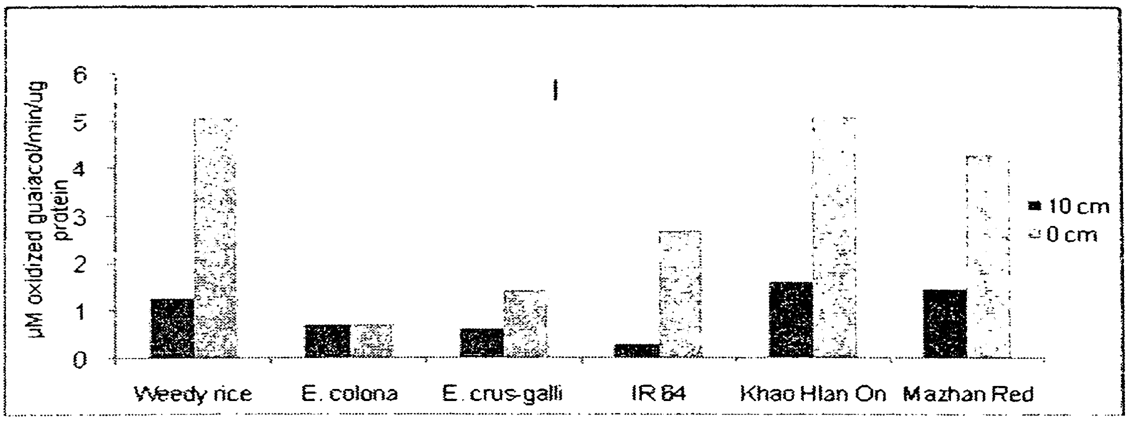

Figure 10. POX activity of weedy rice, E. colona, E. crus-galli and three rice genotypes with different flooding depth at 4 DAS. Vertical bar represents the LSD$_{.05}$.

Table 1. Correlation coefficients for the associations among the MDA content, SOD, CAT, POX, APX and GR activity.

	MDA	SOD	CAT	POX
MDA	1			
SOD	-0.79***	1		
CAT	-0.60**	0.62**	1	
POX	-0.73***	0.84***	0.61**	1
APX	-0.47*	0.56*	0.32ns	0.74***

Note: ***, **, * significant at $P \leq 0.001$, $P \leq 0.01$ and $P \leq 0.05$ respectively, ns = not significant. MDA, Malondialdehyde; SOD, Superoxide dismutase; CAT, Catalase; POX, Guaiacol peroxidase; APX, Ascorbate peroxidise

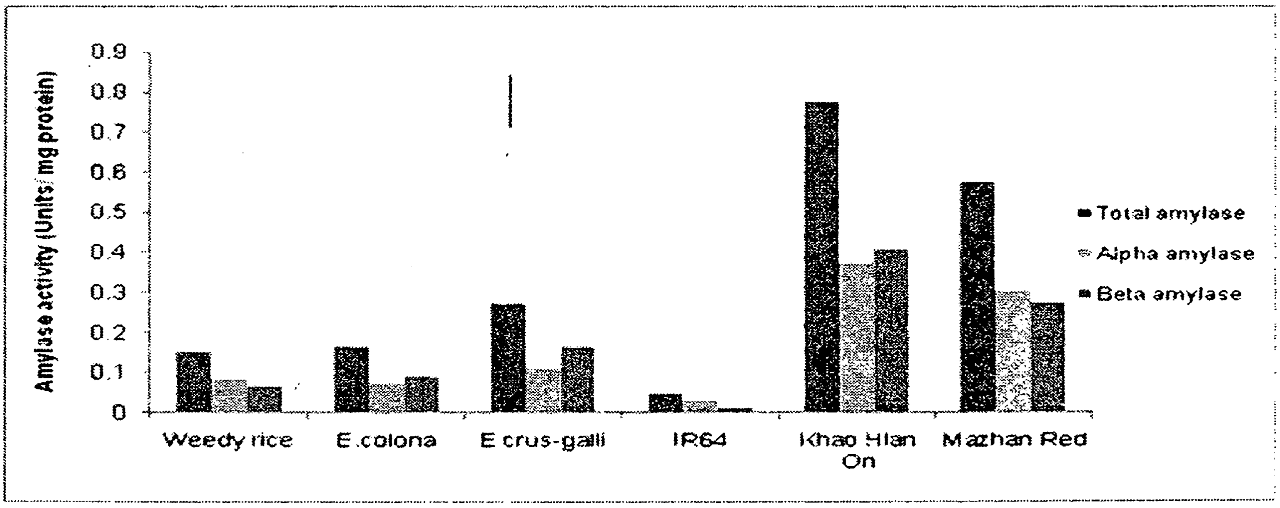

Figure 15. Activity of Total, alpha (α) and beta (β) amylase of weedy rice, *E. colona, E. crus-galli* and three rice genotypes with 10 cm flooding at 4 DAS.

Note: Vertical bar represents the LSD$_{.05}$.

CONCLUSION

Early flooding to 5 cm and 10 cm reduced seedling emergence, shoot and root growth, and biomass of weedy rice, *E. colo*na and *E. crusgalli* indicating that flooding is a viable weed management option against these weeds. Early flooding did not reduce growth of the rice genotypes; KHO and MR. But early flooding reduced growth of IR64 which is, considered to be a flood-intolerant genotype. Data from our studies can contribute to the understanding of the physiological response mechanisms to flooding and help in developing flood-tolerant rice cultivars as well as in developing innovative approaches in managing weeds in wet-seeded rice. This study highlighted the use of tolerant rice genotypes to ensure the crop establishment under flooding conditions and management of weedy rice and *Echinochloa* spp using the early flooding in wet direct seeded rice.

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