Changes in leaf gas exchange, antioxidant enzymes and growth responses in *Jatropha curcas* L.: its relation to waterlogging and recovery

Krishan Kumar Verma¹, Munna Singh¹* and Chhedi Lal Verma²

¹Department of Botany, University of Lucknow, Lucknow-226 007 (UP), India.
²Central Soil Salinity Research Institute, Regional Research Station (CSSRI - RRS), Lucknow – 226 005 (U.P.), India.

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**ABSTRACT**
The responses of photosynthetic gas exchange and chlorophyll fluorescence along with changes in growth were observed in *Jatropha curcas* L. seedlings subjected to waterlogging. The growth characteristics, electrolyte leakage, photosynthetic CO₂ assimilation rate, stomatal conductance and transpiration rates were determined. The activities of catalase, ascorbate peroxidase, glutathione reductase and glutathione peroxidase in leaves increased with the increase duration of waterlogging, implying an integrated pathway involving catalase, ascorbate peroxidase, glutathione reductase and glutathione peroxidase for protection against the detrimental effects of activated oxygen species under waterlogging, but decreased in the recovery period. A strong reduction in photosynthetic and growth characteristics was observed as a results of waterlogging. Decrease in leaf area expansion and stomatal conductance seemed to be the main cause for impairing photosynthesis-carbon assimilation, linked with biomass yield eventually. Further, the ratio between variable to initial chlorophyll fluorescence and the maximum quantum yield efficiency of photosystem II explored damage to the photosynthetic apparatus. Strong nonlinear correlation between physiological parameters and duration of waterlogging was observed.

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**Introduction**
Fossil fuel reserves over the globe are decreasing and global prices soaring continuously putting tremendous pressure on developing economies. Alternate sources of fossil fuel are being searched by the researchers (Verma et al., 2012; 2013). *Jatropha curcas* is one species that has received much attention recently for the production of plant oils that can be converted into biodiesel (Fairless, 2007; King et al., 2009): not only as an energy plant, but also as a drought-tolerant plant used to describe land of poor quality (King et al., 2009; Wang et al., 2011).

*Jatropha curcas* is recommended to be grow abandoned land out of cultivation, more than 12 naha of land in waterlogged in India and lying unproductive since long. *Jatropha curcas* plantation on waterlogged soil may be an economic bi-able option if it grows well.

Waterlogging is one of the major abiotic stresses, which imposes restriction in gaseous diffusion in plants. The slow rate of gas diffusion in water limits the oxygen supply (Visser and Voesenek, 2004; Tan et al., 2010; Verma et al., 2012; 2013).

It has a dramatic impact on biochemical activities, i.e. aerobic respiration and photosynthesis (Armstrong and Drew, 2002; Tan et al., 2010). It damages many agricultural crops and also poorly adapted plants towards natural environments (Jackson, 2004; 2006; Else et al., 2009). Waterlogging of flood water, especially if stagnant or slow moving, is highly damaging to the majority of plant species and can prove fatal (Jackson, 2008). The soil is considered to be waterlogged if free standing water on the soil surface available at least 20% higher than the field capacity (Aggarwal et al., 2006), leads to inefficient supplies of oxygen to the root cells, and this has injurious consequences for the shoot cells, the fundamental requirements for plants’ life. Waterlogging results in major changes in the soil environment, the physical status of the soil, i.e. the break down of large aggregates into smaller particles (Pociecha et al., 2008), a severe threat for survival of terrestrial plants. Waterlogging due to excess rains or seepage from large conveyance and reservoirs after the establishment of the crop, may even damage the crop completely (Islam et al., 2008).

The most important effects are reduction in water and nutrient uptake and disturbances in the plant respiratory metabolism (Dat et al., 2004). It creates O₂ deprivation, which induces several physiological and biochemical changes in shoot and roots that have been well characterised in many plants (Jackson et al., 1996). Oxygen is an essential substrate for respiratory metabolism, passes rapidly through membranes to all compartments of the cell and acts as substrate or cofactor in many biochemical reactions in primary and secondary metabolism of plants (Holmberg et al., 1997).

Roots are major sensory organs for detecting stressful conditions in the soil (Jackson et al., 1996). The shortage of oxygen in rhizosphere becomes detrimental for the development of root systems, and root may eventually die (Bradford and Yang, 1980; Drew, 1997). Root growth is reduced mainly because of the lack of oxygen available to root respiration and presence of soil phytotoxins, inhibiting root formation and promoting root decay. High levels of antioxidant enzymes including catalase, peroxidase, glutathione reductase and ascorbate peroxidase are very important for the survival under oxidative stress of many plants (Tan et al., 2010). The main adverse effects of waterlogging are inhibition of leaf growth, reduction in shoot-root growth and whole plant biomass (Pociecha et al., 2008), changes in biomass partitioning and promotion of overall plant senescence and mortality (Pezeshki, 1994; 2001; Mielke et al., 2003). According to Kozlowski
(1997), shoot growth is reduced because waterlogging affects leaf development, area expansion and induces premature leaf senescence-abscission. The chlorophyll fluorescence is an efficient tool for detecting changes in functioning photosynthesis apparatus, which can be damaged by waterlogging (Mielke et al., 2003; Pociecha et al., 2008). Reductions in whole plant biomass are directly related to changes in net carbon assimilation that are attributed to stomatal and non-stomatal limitations of photosynthesis (Pezeshki, 2001; Mielke et al., 2003; Pociecha et al., 2008). Our aim was to examine responses of *Jatropha curcas* seedlings subjected under waterlogging and recovery condition.

**Materials and methods**

**Plant material and growth conditions**

The forty-five days old seedlings of *Jatropha curcas* L. were raised from stem cuttings (~18-20 cm height) in an open lown area in College of Basic Sciences and Humanities, Pantnagar (Uttarakhand), India, in earthen pots (~30 cm diameter and 40 cm depth) filled with fertile soil. Two water regim es were created simply by changing water application mode. The most favourable moisture regime was created by maintaining soil moisture nearly at field capacity by daily irrigation and the most disadvantageous moisture regime was crated by continuous waterlogging, i.e. maintaining 5 cm of standing water throughout.

**Leaf gas exchange measurements**

Photosynthetic CO$_2$ assimilation, stomatal conductance and transpiration rate were measured by an open system CIRAS-1 portable IRGA photosynthesis system (PP System, England) in natural sunlight (9:00-10:00 h) at photosynthetic photon flux density >1500-1900 µmol m$^{-2}$s$^{-1}$ to avoid high temperature and low humidity in the afternoon. All measurements were taken on mature and fully expanded leaves. Leaf chlorophyll content (SPAD value) was measured by using Chlorophyll Meter (SPAD-502, Minolta, Japan) according to Tan et al. (2008). The chlorophyll fluorescence was assessed by using a handy plant meter apparatus, which can be damaged by waterlogging (Mielke et al., 2003; Pociecha et al., 2008). The most favourable moisture regime was created by maintaining soil moisture nearly at field capacity by daily irrigation and the most disadvantageous moisture regime was crated by continuous waterlogging, i.e. maintaining 5 cm of standing water throughout.

**Antioxidant enzymatic activities assays.**

The catalase activity was measured according to Beers and Sizer (1952) with minor modifications. The reaction mixture (1.5 ml) consisted of 100 mM phosphate buffer pH 7.0 (100 mM), EDTA (0.1 µM), H$_2$O$_2$ (20 mM) and enzyme extract (50 µl), monitored at 240 nm and quantified by its molar extinction coefficient (36 M$^{-1}$cm$^{-1}$) and the results expressed as µmol H$_2$O$_2$ min$^{-1}$ g$^{-1}$ FM. APx activity was measured as per the procedure described by Nakano and Asada (1981). The reaction mixture (1.5 ml) contained 50 mM phosphate buffer, pH 6.0 (50 mM), EDTA (0.1 µM), ascorbate (0.5 mM), H$_2$O$_2$ (1 mM ) and enzyme extract (50 µl). The reaction started by adding H$_2$O$_2$, and ascorbate oxidation measured at 290 nm for 2 min. The enzyme activity quantified using the molar extinction coefficient for ascorbate (2.8 mM$^{-1}$ cm$^{-1}$) and the result expressed in µmol H$_2$O$_2$ min$^{-1}$ g$^{-1}$ FM.

GPX activity was determined as described by Urbanek et al., (1991). The reaction mixture (2 ml) contained 100 mM phosphate buffer pH 7 (100 mM), EDTA (0.1 µM), guaiacol (5 mM), H$_2$O$_2$ (15 mM) and enzyme extract (50 µl). The addition of enzyme extract started the reaction. The increase in absorbance was recorded at 470 nm for 2 min. The enzyme activity was quantified by the amount of tetraguaiacol formed using its molar extinction coefficient (26.6 mM$^{-1}$ cm$^{-1}$). The results were expressed as µmol H$_2$O$_2$ min$^{-1}$ g$^{-1}$ FM. GR activity was measured as described by Foyer and Halliwell (1976), with minor modifications. The reaction mixture (1 ml) consisted of phosphate buffer pH 7.8 (100 mM), EDTA (0.1 µM), guaiacol (2.8 mM), H$_2$O$_2$ (100 mM), ascorbate (0.5 mM), GSSG (3 mM) and enzyme extract (50 µl). The reaction was started by the adding GSSG, and NADPH oxidation monitored at 340 nm for 2 min. The enzyme activity determined using the molar extinction coefficient for NADPH (6.2 mM$^{-1}$ cm$^{-1}$) and expressed as µmol NADPH min$^{-1}$ mg$^{-1}$ FM.

**Growth parameters**

Growth parameters were recorded during waterlogging and recovery (28 days each) period after drained out excess water. Plant height was measured from starting point of the stem to basal leaf (Wielgolaski, 1999) and stem diameter was measured at ~15 cm above the soil surface with a calliper. The leaf areas expansion was estimated by using Leaf Area Meter CI-202 (CID Inc., USA).

**Statistical analysis**

The experiment design was a completely randomized comparing two levels of water in the soil (waterlogging and normal). Photosynthetic and growth characteristics were analysed independently for each evaluation and standard error of means (SE).

**Results**

During waterlogging drastic reduction in plant height (PH), stem diameter (SD) and root length (RL) were observed and a slow recovery after the stress was over. The reduction was ~20, 26 and 68% of PH, SD and RL after 28 days of continuous waterlogging as compared to respective control values (Fig. 1A). As soon waterlogging was over after 28 days recovery phase started. Plant recovered and left over percent reduction in PH, SD and RL were only ~3, 7 and 7% as compared to control values (Fig. 1a). Similar trends were observed in number of leaves (LN), leaf area expansion (LA) and leaf mass per unit area (SLW) during waterlogging and respective reduction were ~58, 64 and 38% (Fig. 1B). LN, LA and SLW increased by about 8,
20 and 36% after 28 days of recovery in relation to control plants (Fig. 1b).

Figure 1. Temporal changes in PH-plant height, SD-stem diameter, RL-root length and LA-leaf number, LA-leaf area, and SLW-leaf mass per unit area of *Jatropha curcas* seedlings grown during waterlogging (A, B) and recovery (a, b) for a period of 28 days. Values are means ±S.E. for five plants.

A reduction in photosynthetic CO₂ assimilation, transpiration rate and stomatal conductance was observed to be ~27, 38 and 24% of respective values of seven days of waterlogging and by the end of 28 days of waterlogging the reduction was ~66, 67 and 45% (Fig. 2A). Similarly photosynthetic gas exchange increased by ~14, 13 and 10% compared to control values after 28 days of recovery period.

Plants under waterlogged conditions showed faster decline in photosynthesis CO₂ assimilation, transpiration rate and stomatal conductance recovered well after 28th days of recovery period (Fig. 2a). A significant reduction in variable to maximum chlorophyll fluorescence (Fv/Fm), and variable to initial fluorescence ratio (Fv/Fo) were observed under waterlogging. 28 days after waterlogging decreased Fv/Fm and Fv/Fo to the tune of 17 and 42%. During 28 days of recovery period, Fv/Fm and Fv/Fo increased by ~4 and 19% as compared to control plants (Fig. 2B and b). Waterlogging induced visible damage was more pronounced in leaves. Waterlogging of 28 days, reduced leaf chlorophyll content (SPAD value) and total chlorophyll was ~18 and 52%, respectively and after 28 days of recovery, leaf chlorophyll content was increased by ~11 and total chlorophyll by ~8% as compared to control plants (Fig. 3B and b). Electrolyte leakage significantly increased in leaf and root of ~23 and 66% after 28 days of waterlogging (Fig. 3A). The electrolyte leakage gradually decreased during 28 days of recovery. No electrolyte leakage was observed in leaf and only 4% leakage was detected in root after 28th days of recovery period (Fig. 3a).

Figure 3. Changes in EC-electrolyte leakage (leaf and root), SPAD-leaf chlorophyll content, Chl. a+b-total chlorophyll in *Jatropha curcas* plants an imposed by waterlogging (A, B) and recovery (a, b) for a period of 28 days. The values represent means (±S.E.) for five plants.

Ascorbate peroxidase and glutathione peroxidase activity increased in response to waterlogging was found to be ~54 and 20% higher than control plants. Left out recovery of ~14 and 5% were marked at the end of 28 days of recovery period in APx and GPx activity levels (Fig. 4A and a). Catalase and glutathione reductase activity increased under waterlogging was observed to be ~32 and 50%. During 28 days of recovery period, CAT and GR activity reached almost to control values. About 4 and 10% of reductions were still left out even after 28 days of recovery (Fig. 4B and b).

Strong correlation were observed between plant height, stem diameter, root length, leaf number, leaf area expansion, leaf mass per unit mass, photosynthetic CO₂ assimilation rate, transpiration rate, stomatal conductance, chlorophyll variable per maximum yield, ratio variable to initial fluorescence, electrolyte leakage in
leaf and root, leaf chlorophyll content, total chlorophyll, ascorbate peroxidase, glutathione peroxidase, glutathione reductase, catalase and number of days of waterlogging. T in the regression equation is time in days. Second order polynomial (quadratic equation) fitted best with the observed data and R² ranged from 0.5655 - 1.000 under waterlogging conditions and 0.9527 - 0.9989 during recovery period.

Where β is the integration constant and can be evaluated by substituting initial conditions in Equation (4) i.e. T=0, R₀ = Rₒ.

\( R_t = \frac{\alpha}{2} T^2 + \alpha \lambda T + R_0 \)  

Equation (4) can now be written as under.

Where R₀ is initial photosynthetic response. Equation (6) is a quadratic equation and non-linear in nature. Regression analysis of percent reduction or increase in physiological response data of plant height, stem diameter, root length, leaf number, leaf area expansion, leaf mass per unit area, photosynthetic CO₂ assimilation, transpiration rate, stomatal conductance, chlorophyll fluorescence variable per maximum yield, ratio variable to initial fluorescence, electrolyte leakage in leaf and root, leaf chlorophyll content, total chlorophyll, ascorbate peroxidase, glutathione peroxidase, glutathione reductase and catalase activities with time period under waterlogging or after waterlogging shows a strong correlation. Second order polynomial i.e. quadratic equation fitted best with the observed data and R² ranged from 0.5655-1.000 under submerged conditions and 0.9527 - 0.9989 during recovery period. The hypothesis of rate of change of percentage decrease or increase in physiological responses under stress and after stress conditions is directly proportional to escalated time period is well verified.

**Discussion**

The inhibition of vegetative growth observed in the experiment confirms earlier results (Shi et al., 2007; Pociecha et al., 2008; Bai et al., 2010). Present observations seem to be in accordance with the opinion of Bacanamwo and Purcell (1999) and Pociecha et al., (2008). The loss in root growth occurs due to waterlogging typically making soil anaerobic (Ponnamperuma, 1984). The soil hypoxia and subsequent anoxia result from biological consumption of oxygen without effective replacement, because the flux of oxygen into the soil is 320,000 times less when soil pores are filled with water compared to conditions when pores are filled with gas (Armstrong and Drew, 2002). Hence, O₂ deficiency in waterlogged soil influenced plant root growth directly; may eventually also down regulate the shoot growth-development. Further, waterlogging also enhances to accumulate CO₂, ethylene, Mn⁴⁺, Fe³⁺, S²⁻ and carboxylic acids (Mc Kee and Mc Kevlin, 1993; Greenway et al., 2006) and associated with the down-regulation of growth and development because, soil flooding induced changes in the level of several plant growth regulators i.e., a decrease in gibberellin and cytokinin along with increase in abscisic acid and ethylene (Bradford and Yang, 1980; Jackson, 2002). Thus, the older leaves can be more strongly damaged after waterlogging because of their high susceptibility to the effect of ethylene. Accordingly, retardation in leaf number and leaf area expansion occurred in response to waterlogging, regarded as a symptom of the acclimation of plants to enable them to avoid the water deficit in leaves, with an early manifestation of injuries as induced by waterlogging similar to the opinion of Bacanamwo and Purcell (1999) and Pociecha et al. (2008). Decrease in biomass and limited leaf area expansion appear to be related slow down metabolic activities of hypoxia roots (Mielke et al., 2003; Yiu et al., 2011) and associated carbon economy based on photosynthetic CO₂ assimilation, as regulated by transport tissue under source to sink phenomenon link with xylem and phloem functionality as well (Cherif et al., 1997; Bai et al., 2010).

The waterlogging influences loss in cellular oxygen content due to decline in photosynthetic CO₂ assimilation based on CO₂
deficiency inside the leaves (Pociecha et al., 2008) due to loss in stomatal conductance. The loss in stomatal conductance favoured internal CO₂ deficiency in leaves during waterlogging, nearly similar to the trends of photosynthesis and transpiration (Souza et al., 2011; Verma et al., 2012; 2013). The loss in stomatal conductance also promotes loss in CO₂ assimilation due to inadequate availability of CO₂ to get assimilated into the biomolecules with the help of Rubisco enzyme. The decrease in stomatal conductance under waterlogging conditions should also be co-related with the decrease in root permeability and root hydraulic conductivity (Mielke et al., 2003), because low stomatal conductance helps to prevent excessive water loss by transpiration to maintain positive water balance (Kozlowski, 1997; Pezeshki, 2001). The rapid stomatal closure has been considered as a waterlogging tolerance mechanism that enhance survival rate under waterlogging from physiological dryness (Pociecha et al., 2008).

The chlorophyll fluorescence is an efficient tool to detect the changes in functioning of photosynthetic apparatus during waterlogging (Mielke et al., 2003; Pociecha et al., 2008). Fv/ Fo has a high power of discernment under the influence of any stress (Babani and Lichtenhaler, 1996; Rohaek, 2002). The decrease in Fv/ Fm and Fv/ Fo ratio suggest loss in photosynthesis due to damage of photosynthetic apparatus (Tan et al., 2008). The Fv/Fm values also indicate response of photosynthetic apparatus during waterlogging, not conducive to achieve higher Fv/ Fm values may be linked with photoinhibition of mesophyll cells (Ahmed et al., 2002a). Waterlogging also caused decrease in leaf chlorophyll content (associated with loss in a/b content), which can be also physically verified with the marked effect of less green (yellow green) leaves similar to Smethurst and Shabala (2003). Zhou and Lin (1995) also reported that waterlogging causes loss in chlorophyll content at various growth stages with the remarks that the most significant changes occurred in lowest leaves. It also suggested that degradation of chlorophyll proceeds more intensively in those leaves that are closure to waterlogged roots. Boru et al., (2001) have also reported leaf chlorosis during waterlogging and also favoured it as a measure of waterlogging tolerance.

Degradation of chlorophyll proceeds more intensively in the older-mature leaves, located very close to the waterlogged roots with chlorosis in Pea and Maize (Przywara and Stepniewski, 1999) and also in Lucerne (Pociecha et al., 2008), as one of the measure to tolerate waterlogging upto certain extent (Boru et al., 2001). Under waterlogged conditions electrolyte leakage was detected in the leaf and root tissues. It appears that waterlogging damaged leaf as well as root membranes. It was more stringent in root compare to leaf tissue. It may be due to limitation of transportation distance from root to shoot to leaves (Amilsuthian et al., 2003).

The involvement of oxidative stress in waterlogging induced damage and the antioxidant response as indicative of tolerance or sensitivity have been studied (Keyhani et al., 2006; Wang and Jiang, 2007) showing a direct relationship between an increased antioxidant activity and stress tolerance (Arbona et al., 2008). The enzyme activities for all these enhanced in support of seedlings survival associated with the stress protection (Arbona et al., 2008; Bailey-Serres and Voesenek, 2008). To control of reactive oxygen species and to protect cells under stress conditions, plant tissue contain several enzymes scavenging reactive oxygen species (superoxide dismutase, catalase, peroxidase and glutathione peroxidase) and a network of low molecular mass antioxidants (ascorbate, glutathione, phenolics and tocopherols). In addition, an array of enzymes is also need for the regeneration of the active forms of the antioxidants (monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase) as reported by Blokhina et al., (2003), judged by cycloheximide (80s ribosome protein synthesis inhibitor) treatment, could not be attributed to de novo synthesis (Biemelt et al., 2000). Amor et al., (2000) has also showed that anoxic pre-treatment protected soybean cells from H₂O₂ induced cell death, associated with up-regulation of catalase, peroxidases and alternative oxidases. The ascorbic acid is powerful antioxidants, detected in plant cell types, organelles and in apoplast (Smirnoff, 2000). Ascorbic acid can directly detoxify superoxide, hydroxyl radicals and singlet oxygen and also reduces H₂O₂ to water via ascorbate peroxidase reaction (Noctor and Foyer, 1998). In inhibition of GR, APx, CAT and SOD activities also occurred as reported by Yan et al., (1996) in corn leaves under prolonged waterlogging, while short term waterlogging led to an increase in the activities. Numerous investigations have demonstrated that the cellular injury to plants by abiotic stresses is oxidative damage (Bowler et al., 1992; Bai et al., 2010). It is now evident that hypoxia tolerance in most plants is associated with a more efficient antioxidant system (Garnczarska, 2005). SOD, POD and CAT are the most important detoxifying enzymes, which work together with APx and GR of the ascorbate-glutathione cycle to promote the scavenging of ROS (Hernandez et al., 2001; Molassistis et al., 2006). In this study, during waterlogging period, the activities of CAT, APx, GPx, and GR against ROS increased in *Jatropha curcas*. This result is in agreement with the reports on the dynamics of these enzymes under chilling (Clare et al., 1984) and drought (Pastori and Trippi, 1993). Inhibition of GR, APx, CAT and POD activities was shown by Yan et al., (1996) in corn leaves under prolonged waterlogging, while a short-term treatment led to an increase in the activities. This early rise of enzyme activities was considered to be the response to increased generation of ROS caused by hypoxia (Bai et al., 2010; Sairam et al., 2011).

In conclusion, the different photosynthetic responses of *Jatropha curcas* found between the normal and stressed conditions would be useful to evaluating of the waterlogged tolerance at the level of individual plant species. From the enzymatic protective mechanism, our data are consistent with an integrated pathway involving CAT, APx, GPx and GR activities for protection against detrimental effects of activated oxygen species under stress. The effects are fully reversible after the stress is relieved in long-term.

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