Effect of Waterlogging Stress on Growth Characteristics and Sod Gene Expression in Sugarcane

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Abstract- Two sugarcane varieties, V1 (an early maturing) and V2 (a mid-late maturing) were planted during spring season of 2014 in tray culture conditions at IMS Engineering college, Ghaziabad with an objective to study the effect of short term waterlogging on growth and physio-biochemical attributes, RNA isolation and expression of SOD (superoxide dismutase) using gene specific primer pairs.

After about 60 days of planting, waterlogging stress treatment was imposed to plants by transferring planted pots in a bucket filled with water. Treatments consist of control 48, 96 hr. of waterlogging stress and recovery after 2 days of termination of treatments. Observations were recorded for SPAD Reading, RWC, chlorophylls and carotenoid contents, proline, and activity of SOD and peroxidase enzymes, soluble protein content and Cu-Zn-SOD gene expression.

Results obtained indicated increased Relative water content, proline content and activity of SOD and peroxidase enzyme under waterlogging duration. An early maturing variety, V1 showed increased SPAD reading and higher accumulation of proline content than variety V2. In both varieties Chlorophylls and carotenoids contents decreased under waterlogging treatment.

Due to waterlogging treatment, in variety V1 SOD gene expression was high at all duration level (0, 48, 96 hr. and recovery) as composed to control, while in V2 variety, treatment plants showed lower expression.

Index Terms- Water logging, SOD, SPAD, RWC, Proline.

I. INTRODUCTION

Sugarcane (*Saccharum officinarum L.*) is an important sugar crops in the world. For high yields, the seasonal crop water requirements for sugar cane crop were estimated at between 1100 to 1500 mm/ha under a range of climatic conditions and varying lengths of growing seasons (12 – 14 months), with a daily evapo-transpiration rate of 4 to 7 mm/day. Using tensiometers in irrigation scheduling (25 – 60 centibars at different crop developmental stages) enables the efficient use of water, fertilizers and energy inputs. Flooding tolerance is related to many physiological and anatomical adaptations of plants. The tolerant species are able to form aerenchyma, which helps for functioning of the plant processes under anoxia conditions (Drew 1997). Glaz *et al*. (2004a) concluded from their study that some genotypes of sugarcane can produce constitutive aerenchyma, meaning that plant requires no external stimulus such as flooding for aerenchyma formation, while some genotypes need exposure to flood to form the aerenchyma. Probably, genotypes having the ability to form constitutive aerenchyma perform better under unpredictable short time flooding conditions, such as caused by heavy rains.

Flooding damage in plant is related to several factors, such as flooding depth, duration and flow of water in the field. However, flooding effects on sugarcane physiology and productivity remain inconclusive. Glaz *et al*. (2004a) and Glaz *et al*. (2002) reported a higher cane yield or unaffected yield, in some of the genotypes they used, in the wetter field (hypoxia). Gascho and Shih (1979) did not find yield differences in two of six cultivars of sugarcane when grown between water table depths of 32-84 cm in lysimeters. Glaz *et al.* (2004b) also noticed a neutral or positive response of gas exchange characteristics of sugacane to flooding. Contrary, Hasan *et al.* (2004), Rahman *et al.* (1986), and Webster and Eavis (1972) reported significant reduction in cane growth under anoxia conditions. Morphological changes in response to flooding were similar in both years, with flooding leading to a 38% reduction in leaf weight, 4 to 15 times greater adventitious root development, 108% greater aerenchyma pipe extension, and 115% greater aerenchyma pipe diameter. Both cultivars responded to flooding by producing aboveground adventitious roots at the expense of belowground primary root biomass.

II. MATERIALS & METHODS

RELATIVE WATER CONTENT (RWC)

20 leaf discs from the leaf of a single sample were punched out. These discs were weighed and the fresh weight was noted down. The discs were immersed in 20 ml of distilled water poured in a petri plate. Steps 1-3 were repeated for the entire second sample. The discs were left in water overnight at room temperature. The discs were separated from the water with the help of funnel and filter paper. The excess water was gently dried from the discs with the help of filter paper and their turgid weight was taken and recorded. These discs were wrapped in a piece of paper and keep in an oven. After 24 hr., the dry weight of the discs of each variety was taken. RWC was calculated according to the formula.

RWC (%) = [(Fresh Weight- Dry Weight) / (Turgid Weight- Dry Weight)] ×100

CHLOROPHYLLS and Carotenoids ESTIMATION

Fresh leaves from each sample were chopped and 0.5g FW of it was weighed. This amount of fresh weight was ground in 10 ml 80 % acetone and centrifuged at 6000 g at room temperature for 10 min. The color intensity was measured at 663, 645 and 470nm using a spectrophotometer. The amount of chlorophyll a

and b and carotenoids was calculated according to Arnon (1949) using the following formulas:
Chlorophyll a= [12.7 (OD_{663}) -2.69(OD_{645})] x (V/1000x wt. in g)
Chlorophyll b= [22.9 (OD_{645}) - 4.68(OD_{663})] x (V/1000x wt. in g)
Carotenoids = [(1000 x OD_{470} -1.82Chl a - 85.02 Chl. b)/198] x (V/1000x wt. in g)
The results were expressed as mg g^{-1} FW.

PROLINE ESTIMATION

0.2 g fresh leaf tissue was extracted in 2 ml of 3% sulphosalicylic acid and extract was centrifuged. A suitable aliquot was taken in a test tube and 2 ml of Ninhydrin Reagent was added to it. After this 2 ml of glacial acetic acid was added to the tubes. The test tubes were heated in boiling water bath for 1 hour. The test tubes when removed from water bath were kept in ice bath to cool them. 5ml of toluene was then added and color was extracted in toluene. The color was read at 520 nm in a spectrophotometer.

SOD ENZYME EXPRESSION

1.5ml of 1M sodium phosphate buffer at pH7.8 was added to sample tubes and 1 blank tube. 0.15ml of EDTA was added to all tubes. Methionine 0.3ml and then 0.3ml of Na_2CO_3 was added to all tubes. 0.3ml of NBT and 0.3ml of riboflavin was added to all tubes. 0.1ml of aliquot was (leaf/root) added to all tubes. OD was taken with the help of a spectrophotometer at 560 nm at 0 times and after 15 minutes during which fluorescent light treatment was given to all tubes.

PEROXIDASE ENZYME EXPRESSION

5 ml of 1M sodium phosphate at pH 6 was added to blank and sample test tubes. Now 2ml of 5N H_2SO_4 was added to all blank tubes. This acts as a stopping reagent. 1ml of H_2O_2 and then 1ml of para-phenyldiamine was added to all tubes. 0.1ml of aliquot (leaf / root) was added to blank as well as sample tubes. Shaking was done in all the tubes. After 5 minutes 2ml of 5N H_2SO_4 was added in all sample tubes. All tubes were kept at 4^o C for up to an hour. OD was taken at 485 nm using a spectrophotometer.

AGAROSE GEL ELECTROPHORESIS

Boil the contents in an oven. Allow to cool upto 60-65^{o}C and then add to it 5µl of EtBr which acts as an intercalating agent. Pour the gel in the casting unit, place a comb and allow solidifying. Prepare TAE running buffer using 2.5 ml of 50x TAE and making up the volume to 250 ml i.e. 1x concentration. Pour the 1x TAE buffer into the running unit. Once the gel solidifies remove the comb and place the gel along with the casting tray into the running unit. Mix 6x loading dye bromophenol blue with the RT-PCR products and load into the wells. 100 kbp marker is loaded in the first well. Connect the unit to a DC current source and allow the gel to run for around half an hour. The gel is estimated using a gel documentation system using a UV transilluminator.

III. RESULTS & DISCUSSION

SPAD Reading

SPAD Reading increased with waterlogging stress condition in variety V1 while SPAD Reading decreased inV2. Recovery showed reversal to nearly normal chlorophyll content.

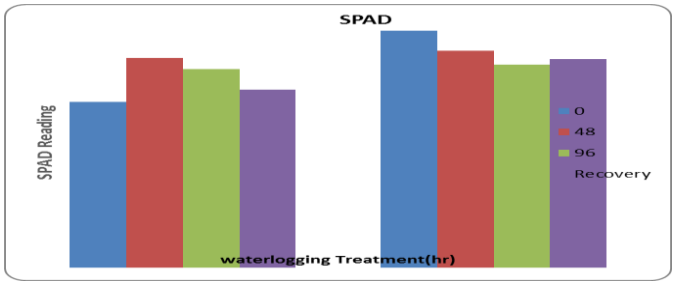


Figure 1: Effect of waterlogging on SPAD Reading

RWC CONTENT

In both the varieties, RWC increased with an increase in waterlogging duration and it ranged from 84.7% to 90.2% in variety V1 and 86.7% to 89.6% in V2.
Recovery treatment showed normal in RWC content.

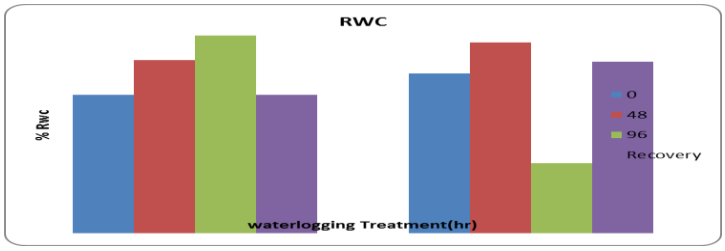


Figure 2: Effect of waterlogging on relative water content

CHLOROPHYLL a CHLOROPHYLL b & CAROTENOIDS CONTENT

Waterlogging treatment decreased photosynthetic pigment Chlorophyll a, Chlorophyll b and Carotenoids content in leaf tissues. Maximum decrease was observed at 96 hour in variety V1and V2.

In Recovery treatment variety V1 did not show further improvement in Chlorophyll a, b and Carotenoids contents indicating severity of waterlogging effect.

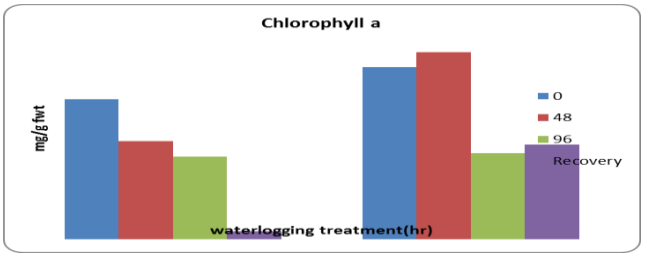


Figure 3: Effect of waterlogging on Chlorophyll a

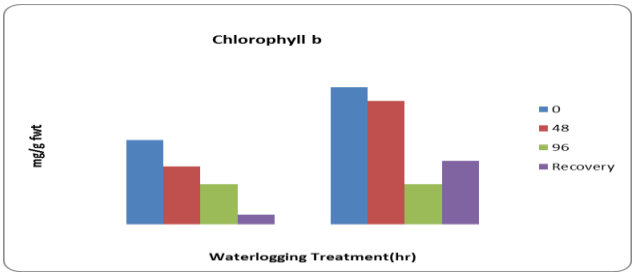


Figure 4: Effect of waterlogging on Chlorophyll b

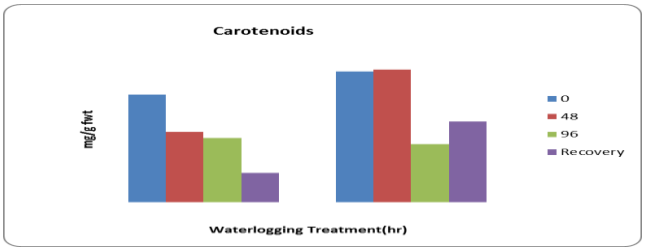


Figure 5: Effect of waterlogging on Carotenoids

PROLINE CONTENT

As compared to control, Proline content in leaf tissues increased with an increase in waterlogging duration, highest increase was observed at 96 hr. waterlogging treatment. Variety V1 Showed higher accumulation of Proline content than variety V2.Further recovery treatment showed reversal to normal Proline.

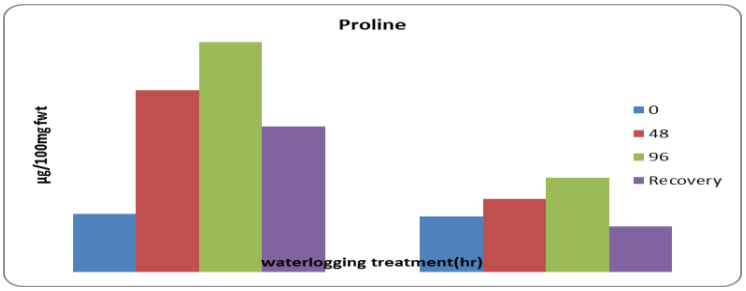
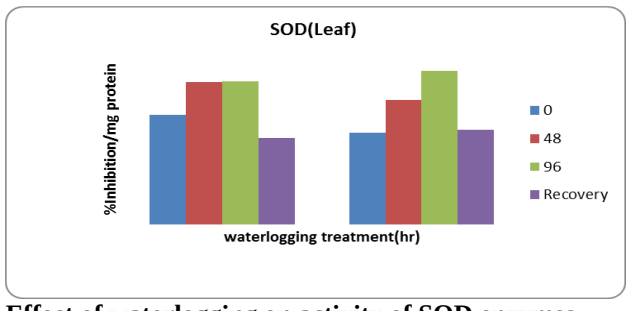


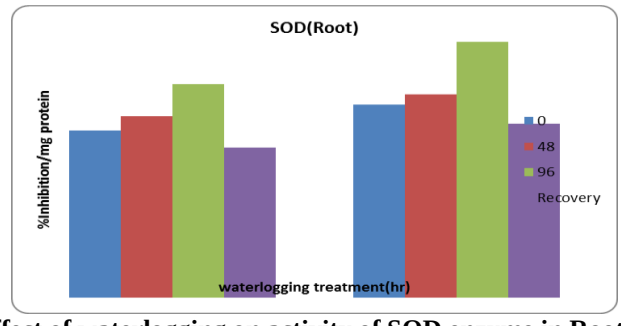
Figure 6: Effect of waterlogging on Proline content

SOD AND PEROXIDASE ACTIVITIES

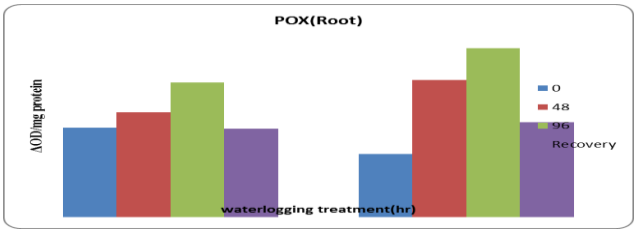
Specific activities of SOD and Peroxidase enzyme was analysed in Root, Leaves under waterlogging stress treatment. In both varieties shows activity of SOD and peroxidase enzymes increased with waterlogging treatment. At 96 hr. increase in SOD and POX activity was very much marked and it was highest in variety V2. Recovery treatment activity of both enzyme decreased markedly 96 hr. waterlogging treatment.



Effect of waterlogging on activity of SOD enzymes.



Effect of waterlogging on activity of SOD enzyme in Root tissues



Effect of waterlogging on Peroxidase enzyme

FIELD EXPERIMENT

SPAD Reading

SPAD reading decreased in leaves of waterlogging affected plants; decrease was about 10.49% in variety V1. While in variety V2, SPAD value increased slightly (6.02% over control) in treatment plants.

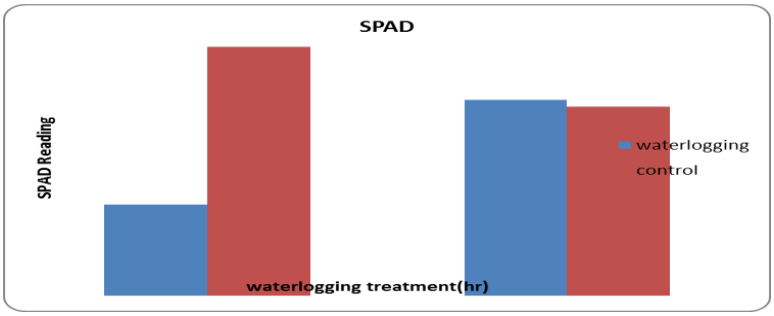


Figure 11: Effect of waterlogging on SPAD Reading.

Cane number

Cane number per clump increased in V1 variety while V2 showed almost equal number of canes in both treated and control plants.

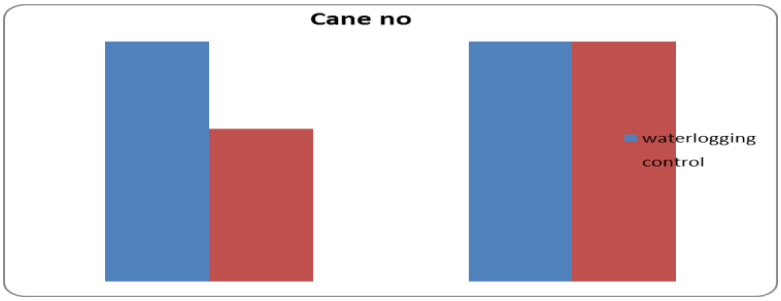


Figure 12: Effect of waterlogging on cane number

Cane height

Cane height increased in both varieties under waterlogging treatment over control. Increase was in the range of 2.95% to 16.94% in variety V1 and V2, respectively.

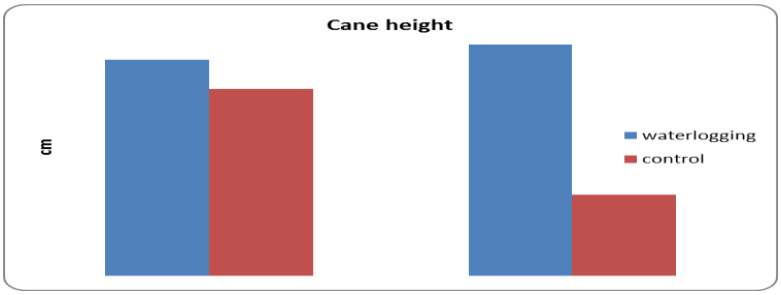


Figure 13: Effect of waterlogging on cane height

Cane weight

In both varieties, cane wt increased in treatment comparatively control but more wt increased in V1 variety. It ranged 212.67% in V1 and 33.47% in V2.

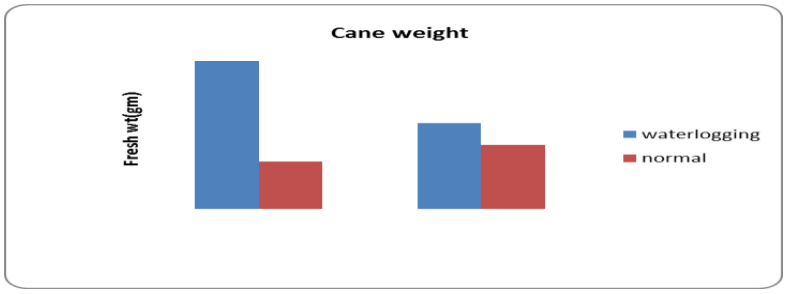


Figure 14: Effect of waterlogging on cane weight

Leaf number

Leaf no increased 104.5% with waterlogging stress condition in variety V1 while it was decreased to 37.73% inV2 respectively than control.

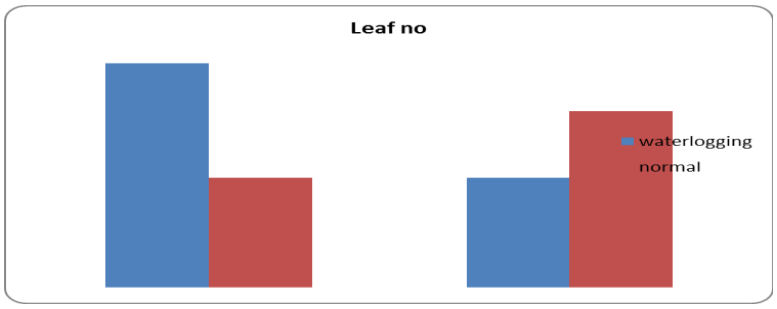


Figure 15: Effect of waterlogging on Leaf number

Leaf area

In both varieties leaf area decreased in waterlogging treatment than control; decrease was 30.67% inV1 and 32.56% in V2 variety.

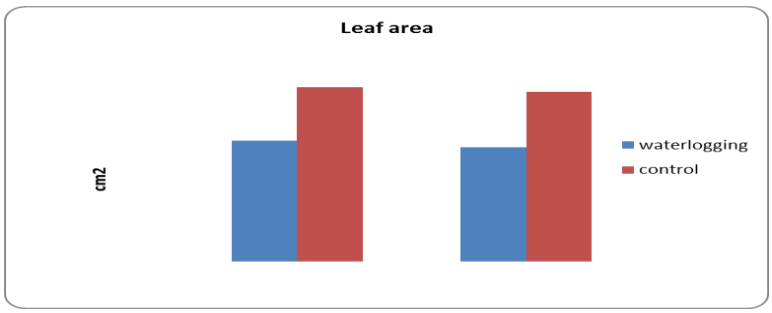


Figure 16: Effect of waterlogging on Leaf area

Leaf weight

Sugarcane plants grown under waterlogged conditions showed increased leaf wt. in variety V1; increase was about 128.85% over control. While in variety V2, it decreased to 24.91% over control.

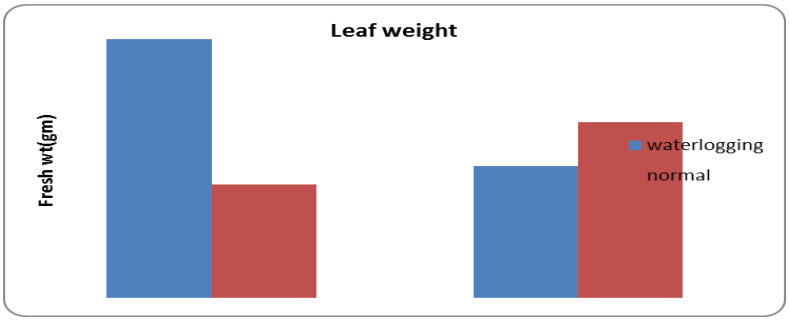


Figure 17: Effect of waterlogging on fresh Leaf weight

Leaf Sheath weight

Leaf sheath weight increased in variety V1 due to waterlogging treatment; increase was about 216.86% over control in V1 and 26.45% leaf weight decreased in variety V2.

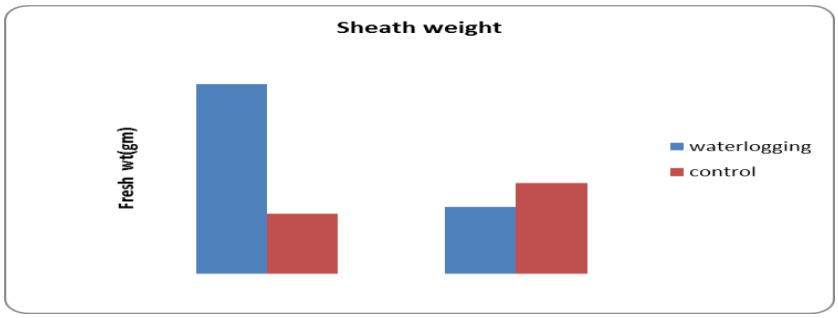


Figure 18: Effect of waterlogging on fresh Leaf sheath weight

Root weight

Increased Root wt. 475.86% with waterlogging treatment in variety V1 than control. In variety V2 it was increased 282.35%.

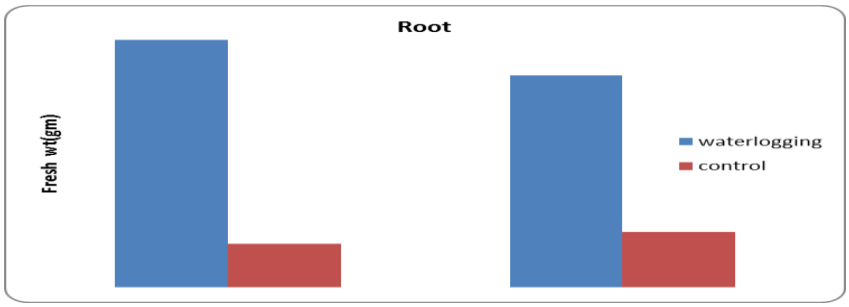


Figure 19: Effect of waterlogging on Root weight

IV. Conclusion

Waterlogging is one of the serious environmental constrain for optimal growth and yield of Sugarcane. Higher rate of stalk mortality, low relative growth rate and reduced cane yield & juice quality loss are major effect of waterlogging. Experiment conducted under net house condition on waterlogging using two sugarcane varieties, V1 (early maturing variety) and V2 (mid late maturing variety). In present study, both cultivars responded to flooding by producing aboveground adventitious roots at the expense of belowground primary root biomass.

Results obtained indicated:

1. Increased proline content in leaf tissues with an increase in waterlogging duration. An early maturing variety, V1 showed higher accumulation of proline content, than variety V2.
2. Variety V1 showed increased SPAD reading with waterlogging treatment while in V2 variety it was decreased.
3. In both varieties, Relative water content increased in waterlogging duration.
4. In both varieties Chlorophyll a, chlorophyll b and carotenoids contents decreased under waterlogging treatment.
5. SOD and peroxidase activity increased due to waterlogging in both the varieties; increase was higher in V2 variety, which helps in tolerance to waterlogging. V2 varieties exhibited relatively tolerance to waterlogging than V1 variety.

6. Studies on SOD gene expression in root through semi qRT-PCR, exhibited higher gene expression in variety V1 at all duration as compared to control,while in V2 variety, treatment plant showed lower expression.
7. In field experiment under waterlogging treatment, cane number, cane height, leaf weight and root weight was increased while leaf sheath weight was decreased. Leaf number increased in variety V1 while it was decreased in variety V2.

APPENDIX

- **80% acetone:** 80 ml acetone added to 20 ml distilled water.
- **Ninhydrin reagent:** 1.259 g Ninhydrin in 30 ml glacial acetic acid, mixed with 6M Phosphoric Acid.
- **6M Phosphoric Acid:** 8 ml Phosphoric Acid + 12 ml distilled water.
- **3% sulphosalicylic acid: 3** g in 100 ml distilled water.
- **50 mM phosphate buffer (pH 7.8):**
- **0.1 mM ethylenediaminetriamine acetic acid (EDTA):**
- **50 mM Na_2CO_3 pH 10.2:**
- **75 µM nitroblue tetrazolium (NBT):**
- **13 mM methionine:**
- **2 µM riboflavin:**
- **0.1 M phosphate buffer, pH 6.0:**
- **0.01% H_2O_2:**
- **0.5% p-phenylenediamine:**
- **5N H_2SO_4,**
- **Reagent A**: 2% sodium carbonate in 0.1N NaOH. From 1N NaOH prepare 0.1N NaOH (10ml 1N NaOH in 90ml distilled water), dissolve 2gm of sodium carbonate into it. Final volume prepared is 100ml.
- **Reagent B:** 0.2gm of sodium tartarate in 10ml of water. Dissolve 0.2gm of sodium tartarate in 10ml distilled water, add 50mg of copper sulphate and mix well.
- **Reagent BC**: Reagent A: Reagent B (200ml:4ml) BC reagent is prepared by mixing reagent A and B in the ratio 200:4, so for 100ml solution mix 100ml reagent A with 2ml of reagent B (100:2).
- **FC Reagent:** As per the required volume, it is diluted three times with distilled water.
- **50 X TAE:** Add 24.2 gm TRIS base ,5.71 ml Acetic Acid and 1.86 gm EDTA to a flask and make up the volume to 100ml

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