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Enhancement of Antioxidant Potential in *Musa Accuminata* using Humic Acid

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Abstract – **Humic Acid is the most important constituent of a completely decomposed organic matter. Leonardite is found to be the best source for Humic Acid and it is extracted by using alkali solution. Humic Acid scientifically proves to be a free radical scavenger. The study determines that Humic Acid (HA) is used to enhance stress tolerance in plant tissue culture. The induction of the antioxidant system [polyphenol, Catalase (CAT), Superoxide dismutase (SOD),Ascorbate peroxidase (APX) and Glutathione S Transferase (GST)] and also the elevation of the content of the enzymes show that the noted changes provoked the treatment of Humic Acid to *Musa accuminata*. Humic acid exposed plants (GWO4 –HU) were found to exhibit only 55 µg of GAE g/dw while native plant (GWO4 – NA) exhibited 147.5 µg of GAE g/dw. The increase of polyphenols in the native may be due to the exposure of roots to the heavy metals that are present in activated charcoal used in rooting medium or due to metal ions in water used. Superoxide anion is one of the most representative free radicals. Both the ethanolic extracts of GWO4-HU and GWO4-NA showed the maximum decolourization at 41.75% and 30.94% at a maximum concentration of 1600 µg/ml with the IC_{50} value 334±0.001 and 247.52±0.050 respectively. In this work, a slight increase in the CAT activity is observed in humic acid exposed plants than native plants. GWO4-HU exhibited 0.042 and GWO4-NA exhibited only 0.038. The raised GST activity is suggested to be a marker for the enhanced antioxidant activity in HA exposed plants. Increased GST activity is found in GWO4-HU of about 557.06 U/mg GST while GWO4-HU exhibit a specific activity of 583.2U/mg GST. APX activity found in GWO4-HU showed higher specific activity of about 408.58 $U.mg^{-1}$ APX while GWO4-NA showed only 379.6 $U.mg^{-1}$ APX.**

Keywords – **Antioxidant Activity, Free Radicals, Humic Acid, Leonardite, *Musa Accuminata*.**

I. INTRODUCTION

The term Humic Acid (HA) represents a group of powerful natural substance that is so complex that science will not be able to replicate them from generation to come. Humic Acids are derived mainly from natural deposits that are highly concentrated, the most common deposits being leonardite (form of oxidized lignite). The presence of insoluble Humic Acid can commonly be found in ordinary soil in lower concentration (0.2% to 10%) [1]. Once raw leonardite are converted into water soluble humates all of the Humic Acid and fulvic acids components will be biologically active and will play an important role in plant and soil stimulation. Humic Acids are not a significant source of plant nutrient but are a soil stimulant and a transportation vehicle for carrying nutrient into plants [1].

Humates have long been used as a soil conditioner, fertilizer, soil supplement. Humus holds not just micronutrient metal ions, but also the essential macronutrients like nitrate, phosphate, and potassium. The significance of Humic Acids is not just limited to its function as a reservoir of mineral plant nutrients and regulator of their liberation [2]. Humic Acid scientifically proves to be a free radical scavenger. Broken molecules are referred to as free radicals that are off – balanced from a point of having excess electrons on them. They cause a great threat to living healthy cells. Humic Acid makes these free radicals safe [3].

Recent research showed that Humic Acid can be used as a growth regulator to regulate hormone level, improve plant growth and enhance stress tolerance [4]. Reactive oxygen species (ROS) are produced by excess transfer energy from triplet excited chlorophyll to oxygen (singlet oxygen formation) or photoreduction of oxygen (formation of superoxide, hydrogen peroxide, and hydroxyl radical) [5]. Excess accumulation of reactive oxygen species results in a series of oxidative injuries to plants [6]–[10].

Oxidative injury involves the initial formation of reactive oxygen species and its subsequent reaction with macromolecules. Proteins, lipids, polysaccharides and nucleic acids can be damaged [5], [7], [11], [12]. As a result, normal cell metabolism can be seriously disturbed. Plants possess intrinsic antioxidant defense mechanisms for coping with reactive oxygen species [13]. Essentially, antioxidant defensive systems fall into three general classes: 1) the lipid- soluble, membrane-associated antioxidants (e.g.-tocopherol, -carotene); 2) the water-soluble reductants (e.g. glutathione, ascorbate); and 3) enzymatic antioxidants (e.g. superoxide dismutase, catalase and enzymes of the ascorbate/glutathione cycle [14], [15]). It has been suggested that the formation of reactive oxygen species is an inherent consequence of metabolism and that control of their levels is essential for normal function. The toxicity of an externally imposed biotic or abiotic oxidative stress can be partly attributed to the overriding of existing resistance mechanisms. Only when those mechanisms are overwhelmed would injury occur [16]. This suggests that the strengthening of the defense mechanisms, through enhancing the functions of their components (such as ascorbic acid, tocopherol, carotene and superoxide dismutase) may reduce or prevent oxidative injury and improve water stress resistance of plants.

Plants are subjected to several environmental stresses that adversely affect the growth, metabolism and yield. Drought is a major abiotic factor that limits agricultural crop production and plant respond to water stress depending on their growth stage as well as the severity and duration ofthe stress. In cellular level, themembrane and proteins can be damaged by reduction in hydration and increase in reactive oxygen species (ROS) [17]. Reactive oxygen species are produced by excess transfer of energy

from triplet excited chlorophyll to oxygen (singlet oxygen formation) or photo reduction of oxygen (formation of superoxide, hydrogen peroxide and hydroxyl radicals). ROS is a dangerous molecule must be detoxified as efficiently as possible to minimize the eventual damage. Excess accumulation of reactive oxygen species results in a series of oxidative injuries to the plant. Plants possess intrinsic antioxidant defense mechanism for copying with reactive oxygen species [18]. Antioxidant enzymes, superoxide dismutase, ascorbate peroxidase and catalase are the efficient scavengers of ROS. Ascorbic acid (AA) is distributed in the cytosol as a water-soluble antioxidant. Dry (and thus sunnier and warmer) conditions have been noted to increase the AA content in turnip greens [19], onions, and black currant [20]. Black currant grown in hot, dry years was found to contain more than twice the AA of those grown in wet years. [21] has reported an enhancement in AA content of plants which were subjected to severe water stress. Research results indicate that, under moderately dry conditions, a slight increase in the total AA concentration occurred. There was an extraordinary upsurge in the total Ascorbic acid (AA) content along with abnormally high oxidation-reduction ratio and very high level of respiration under extreme drought conditions, suggesting a complete breakdown of the metabolism [22]. [23] noted that AA increased significantly under water stress. In contrast, [24] found a decrease of ascorbate content of drought-stressed treatment in 9 out of 10 grass species. Superoxide dismutase (SOD) is closely related to the capability of plant tolerance to various stresses, such as water stress, chilling stress, herbicides, and pathogens [25]-[27]. It was reported that SOD enhances water stress tolerance of plants. In tomato, cytosolic Cu/Zn-SOD (Copper / Zinc – Superoxide dismutase) was induced strongly by drought, while chloroplastic Cu/Zn-SOD remained largely unaffected [27]. In two mosses, the drought-tolerant *Tortularuralis* and drought-sensitive *Cratoneuronfilicinum* were compared [28]. The drought-tolerant moss showed lower levels of lipid peroxidation, together with increased levels of SOD; the opposite occurred in the sensitive moss. Drought tolerant and intolerant maize in breds were analyzed by [29], and resistance was found to correlate with Cu/Zn-SOD. Drought stressed tomato showed increases both in transcript levels of cytosolic Cu/Zn-SOD gene (eight-fold) and enzyme activity (five-fold), whereas an increase in enzyme activity was observed in chloroplastic Cu/Zn-SOD (2.4-fold) without a corresponding increase in transcript levels [30].

II. Material and Methods

Enhancement of Antioxidant Potential in Musa accuminata

A. Estimation of Polyphenols

The amount of polyphenols in the leaf sample (growth regulator supplemented and Humic Acid propagated) was determined by Folin-Ciocalteu's method as described by [31]. Leaves were homogenized in 80% ethanol and centrifuged separately. The supernatant was saved. The residue was again extracted with 80% ethanol. The supernatant was collected and evaporated to dryness. The residue was dissolved in water and 2 ml was taken for the experiment. The volume was made up to 2.5 ml with water and 0.5 ml of diluted Folin- Ciocalteu reagent wad added. After 3 minutes, 2 ml of 20% Na_2CO_3 (Sodium carbonate) was added. Incubate in boiling water bath for 1 minute. Measure OD (Optical Density) at 650 nm. A standard graph was plotted using catechol as standard with the concentration ranging from 50-250µg. The results were expressed as milligrams of Gallic acid equivalent per gram of dry weight (GAE g/dw).

B. Preparation of Enzyme Extract

Frozen leaves are homogenized at 4°C in a pre-chilled mortar and pestle with liquid nitrogen in 100 mm potassium phosphate buffer (pH 7.8), 1mm EDTA, 1% triton x 100, 15% glycerol, with 50 mg of PVP per gram of leaf. Crude homogenates was centrifuged at 15,000 rpm for 15 minutes at 4°C. Supernatant was frozen at -20° C. These enzyme extract is used to determine the protein content by Bradford assay using BSA as standard

C. Estimation of Protein by Bradford Assay

Pipette a standard BSA (Bovine Serum Albumin) 50-250 µl with the concentration of 50- 250 µg and 50 µl of sample in test tubes. The volume was adjusted to 0.250 ml with distilled water. 3ml of Bradford reagent was added and mixed thoroughly; it was then incubated at room temperature for 10 minutes. The optical density was read on spectrophotometer at 595 nm and the readings were recorded. The calibration curve or standard curve was drawn by plotting average optical density reading on Y axis against standard protein concentration on x axis.

The value X from the graph was recorded corresponding to optical density of the test sample.

Using (1), we calculater the protein concentration present in the test sample

Protein concentration in test sample

$$= X/V \ (mg.ml^{-1}) \qquad (1)$$

Where X – Value from graph in µg

Y – Volume of sample in µl

D. Superoxide Dismutase

Superoxide dismutase was generated by xanthine oxidase and detected by nitro blue tetrazolium (NBT) reduction method [32]. Reagents in this study are prepared with 50mM potassium phosphate potassium hydroxide buffer (pH 7.8).

The reaction buffer should contain 50 µl of 0.6 mM NBT, 20 µl of 15 mM Na_2EDTA (Disodium ethylenediamine tetraacetic acid) (pH 7.4), 30 µl of xanthine oxidase solution, 150 µl of enzyme sample and 1.5 µl of potassium phosphate potassium hydroxide buffer is used as control. Reaction was initiated by the addition of xanthine oxidase at 25° C. The absorbance at 405 nm was recorded every 20 seconds for 5 minutes in UV visible spectrophotometer. Using (2), results were expressed as % of inhibition relative to control, given by

$$\frac{\text{Rate of control - rate of sample reaction}}{\text{Rate of control}} \times 100\% \qquad (2)$$

The dosage of extract is expressed in µg of dry weight of the extract per ml of assay mixture. IC_{50} value represents the concentration of test compound where the inhibition test activity reached 50%.

E. Catalase (CAT)

Catalase activity was estimated by the method of [33] with minor modification. The reaction mixture containing 50 mm sodium phosphate buffer (pH 7.0), 20 mm H_2O_2 and 1 ml enzyme sample. Decrease in absorbance was noted at 240 nm. The molar coefficient of H_2O_2 at 240 nm was taken as 43.6 M/cm. The enzyme activity was expressed as µmoles of H_2O_2 degrade minutes/gram.

F. Glutathione S Transferase (GST)

Glutathione S Transferase activity was determined by using IZZ0 AND IZZ0 method [34]. 0.5 gram of plant material was homogenized in 5 ml of 0.1 M of potassium phosphate buffer (pH 7.4) that contains 1Mm of EDTA and 0.2 mM PVP. The extract was centrifuged at 8,000 rpm at 4º C for 20 minutes. 1ml of sample was added to 3 ml of 0.1 M phosphoric acid buffer (pH 7.0) that containing 2mM of CDNB (1-chloro- 2,4 dinitrobenzene) and 2mM of reduced glutathione (GSH). The increase in absorbance was recorded at 340 nm for7 minutes. The molar extinction coefficient of 9.6 mM/cm for CDNB GSH was used and expressed in units of enzyme activity per 1 mg of protein.

G. Ascorbic Acid Peroxidase (APX)

APX activity was determined according to [35]. APX extraction was performed in 1.5 ml of suspension solution including 50 mMTris-HCl(pH 7.2), 2 % PVP (Polyvinylpyrrolidone), 1 mM Na_2EDTA, and 2 mMascorbate. Assay solution contained 50 mM potassium phosphate buffer (pH 6.6), 2.5 mMascorbate, 10 mM, H_2O_2 and enzyme containing 100µg proteins in a final volume of 1 ml. The enzyme activity was calculated from initial rate of the reaction using the extinction coefficient of ascorbate (ε = 2.8 mM cm^{-1} at 290 nm).

III. Results and Discussion

HA enhancing the antioxidant activity of Musa accuminata

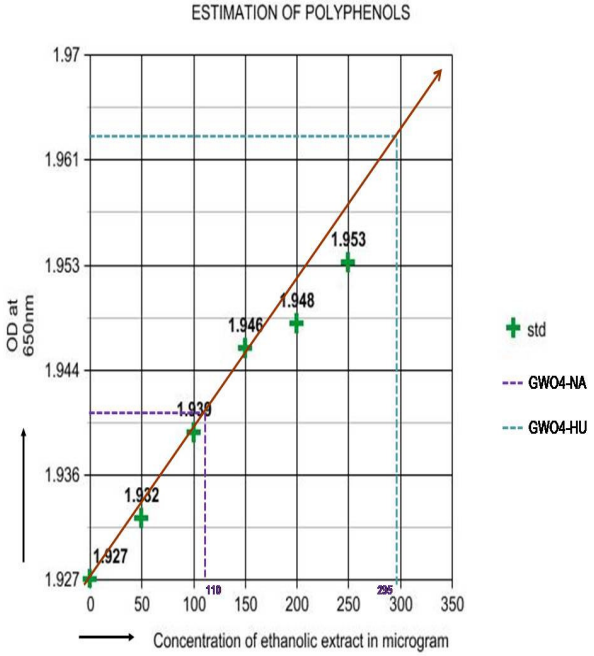
A. Polyphenols

Antioxidant action of phenolic compounds is due to their high tendency to chelate metals. Phenolics possess hydroxyl and carboxyl groups, able to bind particularly iron and copper [36]. Humic Acid exposed plants were found to exhibit only 55 µg of GAE g/dw) while native plant exhibited 147.5 µg of GAE g/dw). The increase of polyphenols in the native may be due to the exposure of roots to the heavy metals that are present in activated charcoal used in rooting medium or due to metal ions in water used. But HA exposed plants found to inactivate iron ions by chelating and additionally suppressing the superoxide-driven Fenton reaction, which is believed to be the most important source of ROS [37]. Direct chelation action of polyphenols is observed in rhizome of Nympheae for Chromium, Lead and Mercury [38].

Table I: Estimation of Polyphenols

Concentration in µg	OD AT 650 nm
50	1.932
100	1.939
150	1.946
200	1.948
250	1.953
GWO4 NA	1.963
GWO4 HU	1.941

The concentration of ethanol extract samples, GWO4 NA and GWO4 HU were found by plotting the graph having catechol as standard. Graph is displayed in graph no.1, the values were found to be 1.963 µg for GW04 NA which indicates the clone for the native plant and 1.941 µg for GW04 HU, which indicates the clone given for humic acid induced plants at 650 nm.



Graph 1: Estimation of Polyphenols

The concentration of GW04 NA (clone given for native plant) is 295 µg/2ml which is 147.5 $\mu g.ml^{-1}$ of GAE g/dw and GW04 HA (clone given for humic acid induced plant) is 110 µg/2ml which is 55 $\mu g.ml^{-1}$ of GAE g/dw.

Humic Acid is found to decrease 2.68 times less polyphenol content when compared with native plants. Thus either stress due to metal ions or environmental factors are reduced predominantly.

Table II: Estimation of Protein by Bradford assay

Concentration in µg	**OD at 595 nm**
50	0.880
100	0.910
150	1.110
200	1.190
250	1.270
GW04 NA	0.981
GW04 HU	1.008

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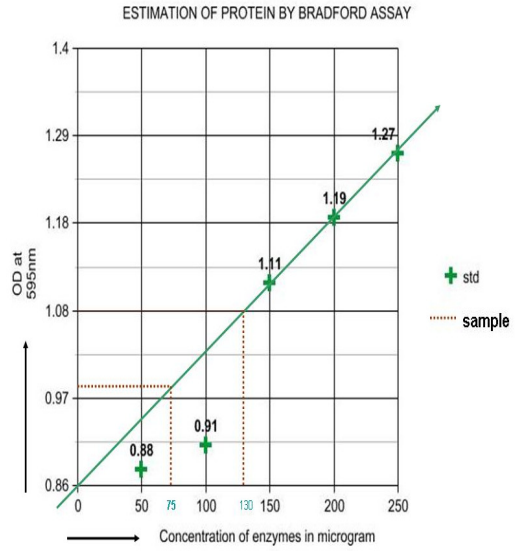
Protein concentrations of GW04 NA, GW04 HU were calculated by plotting the graph having BSA as standard. Graph is displayed as graph no: 2 the values are found to be that GWO4 NA is 75 µg/ 50 µl which is 1.5 mg/1ml and GWO4 HU is 130 µg/ 50 µl which is 2.6 mg/1ml.



Graph 2: Estimation of protein by Bradford assay

The standards using BSA 50 – 250 µg is plotted in graph (2) by obtaining OD values at 595 nm. The OD values for the sample containing native plant and humic acid propogated plants were compared by plotting in the graph. The result obtained proved that Humic Acid propagated plants exhibited 1.73 times more enzyme concentration than native plants.

B. Superoxide Dismutase

Superoxide anion is one of the most representative free radicals. In cellular oxidation reactions, superoxide radicals have their initial effects magnified as they produce oxidizing agents e.g., hydroxyl radicals [39].

The dosage of the extract is expressed as in µg of dry weight of the extract (compound) per ml of the assay mixture. IC_{50} value represents the concentration of test extract or compound where the inhibition of test activity reached 50%. Both the ethanolic extracts of GWO4-HU and GWO4-NA showed the maximum decolourization at 41.75% and 30.94% at a maximum concentration of 1600 µg/ml with the IC_{50} value 334±0.001 and 247.52±0.050 respectively.

The percentage of inhibition is calculated using "(1)". For GWO4 NA the rate of control is 0.782 and the rate of sample reaction is 0.540. Therefore the % of inhibition is calculated and found to be 30.94%. Similarly GWO4 HU the rate of control is 0.673 and the rate of sample reaction is 0.392. Using the formula we get 41.75% inhibition by GWO4-HU.

C. Catalase Activity (CAT)

Catalase is an enzyme that converts hydrogen peroxide to water. This enzyme rapidly destroys a vast majority of H_2O_2 produced in peroxisomes during photorespiration and formed asa result of mitochondrial electron transport, but it allows low steady state levels to persist presumably to maintain redox signaling pathways [40]. In this work, a slight increase in the CAT activity is observed in Humic Acid exposed plants than native plants. GWO4-HU exhibited 0.042 and GWO4-NA exhibited only 0.038. Increase of CAT activity is observed in NaCl treated barley roots. CAT has been observed as the major enzyme detoxifying hydrogen peroxide in barley under salt stress [41].

The Volume activity and Specific activity is calculated using the formula

Volume activity =
[(Total volume) / (Extinction Coefficient x dilution factor x sample)] x [(A / incubation time)] (3)

$$\text{Specific activity} = \frac{\text{Volume activity}}{\text{Protein concentration}} \quad (4)$$

The total volume of GWO4 NA is 3 ml, the molar extinction coefficient of catalase at 240 nm is 43.6 M/cm, dilution factor 1 and 1 ml of the enzyme sample. The volume activity is calculated and found to be 0.057 U/ ml/ sample and specific activity is 0.038 $U.mg^{-1}$ catalase.

For GWO4 HU the Volume activity from "(3)" and "(4)" is calculated and found to be 0.11 U/ ml/ sample and specific activity is 0.042 $U.mg^{-1}$ catalase.

Table III: Enzyme activity of Catalase

Samples	Enzyme Activity ($U.mg^{-1}$ catalase)
GWO4 NA	0.038
GWO4 HU	0.042

Both the normal and Humic Acid propagated plants had no prominent difference incatalase test. Both the values fall very close to each other.

D. Glutathione S Transferase (GST)

The raised GST activity is suggested to be a marker for the enhanced antioxidant activity in HA exposed plants. It is possible that chlorophenols such as CDNB may induce GST directly or indirectly by toxic organic radicals, ROS or through lipid peroxidation. Increased GST activity is found in GWO4-HU of about 557.06 $U.mg^{-1}$ GST while GWO4-HU exhibits a specific activity of 583.2 $U.mg^{-1}$ GST. [42] Noted increased GST activity L. esculentum roots under salinity stress.

Enzyme activity of native and Humic Acid supplemented plants

Volume activity =
[(Total volume) / (Extinction Coefficient x dilution factor x sample)] x [(A / incubation time)] (5)

$$\text{Specific activity} = \frac{\text{Volume activity}}{\text{Protein concentration}} \quad (6)$$

The volume activity of GWO4 NA is 835 U/ ml/ sample and the specific activity is 557.06 $U.mg^{-1}$protein whereas GWO4 HU volume activity is 1516.32 U/ ml/ sample and specific activity is 583.2 $U.mg^{-1}$protein from "(5)" and "(6)".

Table IV: Enzyme activity of GST

Sample	Enzyme Activity $U.mg^{-1}$GST
GWO4 NA	557.06
GWO4 HU	583.20

The enzyme activity of GST in GWO4 HU is higher in comparison to the native plant.

E. Ascorbate Peroxidase

APX has a higher affinity for H_2O_2 (mM range) than CAT and POD (mM range) and it may have a more crucial role in the management of ROS during stress. GWO4-HU showed higher specific activity of about 408.58 $U.mg^{-1}$APX while GWO4-NA showed only 379.6 $U.mg^{-1}$APX. The findings of [43] suggest that cytosolic APX1 plays a key role in protection of plants to a combination of drought and heat stress.

Table V: Enzyme activity of Ascorbate peroxidase

Sample	Enzyme Activity ($U.mg^{-1}$ascorbate peroxidase)
GWO4 NA	379.6
GWO4 HU	408.58

As given in the table above the enzyme activity of Ascorbate peroxidase is higher when compared to the native plant.

III. Conclusion

The results of this study demonstrated that Humic Acid induced tissue cultured plants possessed high antioxidant defense mechanisms for copying with reactive oxygen species when compared to the native plant that is used as a control. The increase in the contents of enzymes, proteins and the induction of the antioxidant system showed noted changes given by the treatment of Humic Acid to tissue cultured Musa accuminata. The polyphenol content is reduced in Humic Acid propagated plants which proved reduced metal ions stress in plants by Humic Acid. The water soluble reductants and enzymatic antioxidants in Humic Acid exposed plants were significantly higher than the native plants by almost two times. The present study proved that Humic Acid enhanced stress tolerance in plants.

Appendix

AA – Ascorbic acid
APX – Ascorbic Acid Peroxidase
BSA – Bovine Serum Albumin
CAT - Catalase
CDNB – GSH – 1-chloro-2,4 dinitrobenzene - glutathione
Cu / Zn – SOD – Copper / Zinc – Superoxide dismutase
EDTA - Ethylenediamine tetraacetic acid
GAE g / dw - Gallic acid equivalent per gram of dry weight
GST – Glutathione S Transferase
HA – Humic Acid
HU – Humic acid induced plants
H_2O_2 – Hydrogen peroxide
IC50 - Half maximal inhibitory concentration
NA – Native plant
Na_2CO_3 - Sodium carbonate
Na_2EDTA - Disodium ethylenediamine tetraacetic acid
NBT - Nitro blue tetrazolium
OD – Optical Density
PVP - Polyvinylpyrrolidone
ROS - Reactive Oxygen Species
SOD - Superoxide dismutase
$U.mg^{-1}$ – Units per milligram
UV – Ultraviolet

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