

In Vitro Growth and Physiology of Wheat (*Triticum aestivum L.*) Seedlings Under Aluminum Stress.

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ABSTRACT: This research was conducted to study the effects of Aluminum (Al) on Wheat seedlings growth and their physiology under *in vitro* conditions. Plant tissue culture techniques were applied to see the growth pattern of Wheat plant in the presence of Al. Wheat cultures were established on Ms Media supplemented with 6-BAP(6-Benzylaminopurine), IAA(Indole-3-AceticAcid)by introducing stable Wheat *in vitro* plantlets. For further study, different concentrations of Al were applied and observed seedlings growth and physiology under Al stress. In the present study, different parameters were studied to see Al stress, and they were, root growth, shoot growth, fresh weight, dry weight, RGI (Relative Growth Index), % of phytotoxicity, chlorophyll and MDA (Malondialdehyde) contents in tissues of Wheat seedlings. The results indicated that the maximum root and shoot growth inhibition occurred at the higher concentrations of Al. The amount of Chlorophyll content also reduced with the Al treatment and resulted in a significant increase in MDA levels, an indicator of lipid peroxidation. The present study results indicate that the growth and physiology of *in vitro* planted seedlings of Wheat were severely affected by the Al toxicity.

KEY WORDS : *In vitro*, 6-BAP(6-Benzylaminopurine), IAA (Indole-3-AceticAcid), RGI (Relative Growth Index), % phytotoxicity, MDA (Malondialdehyde), Lipid Peroxidation.

I. INTRODUCTION

Most of the heavy metals are reported as toxic pollutants for plants, animals and humans. They enter into the environment (soil) from industrial processes and phosphate fertilizers. Ultimately they enter into food chain with potential consequences for human health (Jarup, 2003).Biotechnological efforts are underway to improve plant metal tolerance and ability to extract heavy metals from the soil (Salt *et al.*, 1995). In order to devise new strategies for phyto remediation and improved tolerance, it is important to understand as to how heavy metals are taken up act at cellular and tissue level. (Schut 3 Endubel Polle, 2002: Beak *et al.*, 2006).Al toxicity is the most important factor, being a major constraint for crop production on 67% of the total acid soil area (Eswaran *et al.*, 1997). The primary response to Al stress occurs in the roots (Foy *et al.*, 1978; Foy, 1984, Taylor, 1988, Jayasundra *et al.*, 1988).

The main symptom of Al toxicity is rapid inhibition of root growth. Roots get injured due to Al toxicity and become stubby and brittle. The other symptoms caused by Al are thickening and brown coloration of root tips and lateral roots. The whole root system gets affected with many stubby lateral roots and no fine branching. Such roots are inefficient in absorbing nutrients and water (Foy *et al.*, 1978).

Wheat (*Triticum aestivum L.*) is the most common cereal available as a food throughout the world. It belongs to the botanical tribe *Triticaceae* in the gross family of *Poaceae*. It is higher in demand in recent years due to its abundant health benefits. Previous research findings proved that Wheat is extremely beneficial for healthy living. It is considered to lower hazards of heart disease because of its low fat content. It is also beneficial for diabetic patients due to its capability of regulating blood glucose levels. Wheat has lot of other health benefits; it consists of abundant amount of

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 8, August 2016

vitamins, particularly vitamin E and minerals. It is known to be as a main source of vitamin complex like Thiamin, Folic acid, vitamin B6 and minerals like Manganese and Zinc.

Wheat (*Triticum aestivum L.*) prefers a nearly neutral soil pH (about 6.4) and does best with a cool, moist growing season followed by warm, dry weather for ripening. The affects of Al on Wheat seedlings were studied in the present investigation under *in vitro* growth conditions. The doses of Al i.e. 1.5mM, 3.5mM, 5.5mM, 7.5mM, and 9.5mM were used as a treatments in the present study.

II. MATERIALS AND METHODS

MS medium was used as a basal medium in present investigation. Composition of MS media is given in the following tables. Initially prepared all the stock solutions required for the MS medium for the accuracy and also for time saving purpose.

Composition of MS Medium:

I. Macro stock (MS-I) in 1000 ml.

S. No.	Nutrient Name	Quantity in g (10 × 100)	Volume of Stock (in ml/L)
1.	KNO ₃	19.0	100.0
2.	NH ₄ NO ₃	16.5	100.0
3.	KH ₂ PO ₄	1.7	100.0
4.	MgSO ₄ .7H ₂ O	3.7	100.0

The above mentioned salts were taken into a 2 liter beaker, dissolved initially in 500ml distilled water (H₂O). Finally the volume was made up to 1000ml by adding distilled water. Then this solution was labeled as stock- I solutions and stored at 4⁰c.

II. Calcium stock (MS-II) in 1000 ml.

S. No.	Nutrient Name	Quantity in g (10 × 100)	Volume of Stock (in ml/L)
1.	CaCl ₂ .2H ₂ O	4.4	100.0

4.4g of CaCl₂.2H₂O was taken into a 2.0 liter beaker and then dissolved with 500ml distilled water and finally made up the volume 1000ml by adding distilled water (H₂O). Stored at 4⁰c. This is the Stock – II.

III. Micro stock (MS-III) in 1000 ml.

S. No.	Nutrient Name	Quantity in g (10 × 100)	Volume of Stock (in ml/L)
1.	H ₃ BO ₃	62	100.0
2.	MnSO ₄ . H ₂ O	168.9	100.0
3.	ZNSO ₄ .7H ₂ O	86	100.0
4.	KI	8.3	100.0
5.	Na ₂ MoO ₄ .2H ₂ O*	2.5	100.0
6.	CuSO ₄ .5H ₂ O**	0.25	100.0
7.	CoCl ₂ .6H ₂ O**	0.25	100.0

*Prepared separately and then added

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 8, August 2016

**Prepared 100 mg in 100 ml DDH₂O and then added required quantity (for 1000 ml of 100X-25 ml)
In a 2.0 liter beaker, dissolved all the salts sequentially in a descending order with 500ml of double distilled H₂O and finally made up the volume up to 1000ml by adding distilled H₂O and labeled it as stock- III solution and Stored at 4⁰c.

IV. MS Iron EDTA stock (MS-IV) in 1000 ml

S. No.	Nutrient Name	Quantity in g (10 × 100)	Volume of Stock (in ml/L)
1.	Na ₂ EDTA. 2H ₂ O	3.73	100.0
2.	FeSO ₄ . 7H ₂ O	2.78	100.0

1000ml double distilled water was taken in a 1500 ml of amber colored bottle and warmed the water up to near boiling. Then added Na₂ EDTA. 2H₂O while stirring under magnetic stirrer. After it get dissolved added FeSO₄ gradually and mild stirring was done using magnetic stirrer. Then closed the bottle immediately and kept on stirring for an hour. And then labeled it as MS-IV and stored at 4⁰C.

V. Vitamin stock (MS-V) in 1000 ml.

S. No.	Nutrient Name	Quantity in g (10 × 100)	Volume of Stock (in ml/L)
1.	Myo- inositol	1000	100.0
2.	Glycine	20	100.0
3.	Thiamine HCL	1	100.0
4.	Nicotinic acid	5	100.0
5.	Pyridoxine HCI	5	100.0

500 ml double distilled water was taken in a 2 liter beaker and then added every salt sequentially in a descending order and kept on dissolving the salts using magnetic stirring, and finally made up the solution volume to 1000 ml by adding double distilled water. Later labeled it as MS-V and stored at 4⁰C.

VI. Growth regulator stocks:

Growth regulator (Auxin and Cytokinins) stocks were prepared at a concentration of 1.0 mg/ml.

MS Medium preparation:

Mixing of all stock solutions:

MS Medium was prepared by adding the above said all stock solution in a sequential manner. The below given table gives details about how much quantity of each stock solution is needed to prepare the MS Medium.

S. No.	Stock	Quantity in ml or g per liter.
1.	MS-I	25.0
2.	MS-II	25.0
3.	MS-III	25.0
4.	MS-IV	25.0
5.	MS-V	25.0
6.	Sucrose (gm)	30.0
7.	Calciumdpentatinat e	2.00

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 8, August 2016

Preparation of 1 liter of MS basal Medium:

For preparation of 1 liter of MS Basal Medium, all the above mentioned stock solutions were added sequentially in about 500 ml of double distilled water. And then added 30 g of sucrose and dissolved it with the help of magnetic stirrer. Then IAA and 6-BAP the medium growth regulators were added and volume of the medium was made up to 1000 ml by adding distilled water. pH of the medium was adjusted to 4.5 using 0.1 NaOH or 0.1 N HCL before autoclaving the medium.

Preparation of semi-solid medium:

Different amounts of agar was used (i.e., 8.0g, 9.0g, 11g, 14g, and 22g), because increasing concentration of Al may interfere solidification of medium. Medium was transferred into 6 conical flasks containing different concentrations of Al (1.5Mm, 3.5Mm, 5.5Mm, 7.5Mm, 9.5Mm). And then boil the medium until the agar gets dissolved completely. Later sterilize this medium by autoclaving at dispense the medium aseptically in sterile culture vessels. After the autoclave, the medium was dispensed into the sterile culture vessels. Wait until the medium gets solidify, mean the time, seeds were surface sterilized with HgCl₂ for 3min, followed by washing with double distilled water for several times and Under laminar air flow. And then these seeds were cultures on MS Medium containing different concentrations of Al. Control was maintained along with other treatment tubes except without adding Al. All culture tubes were maintained in a growth chamber at 25^oc, 70% RH and irradiance of 50-60 μmol m⁻² s⁻¹T with 16 hours photo period. All the aseptic conditions were maintained throughout the period of seed germination.

Growth Parameters:

Morphological Changes: - Seedlings were observed for morphological changes. The visual symptoms of toxicity if any were noted on the 8th day.

Root and Shoot length: - The seedlings were separated into roots and shoots and length of each part was measured using a graph paper.

Percent phytotoxicity: - It was calculated as follows:

$$\text{Percent phytotoxicity} = \frac{\text{root length of control} - \text{root length of test}}{\text{Root length of control}} \times 100$$

Dry weight: - The seedlings were separated into roots and shoots, gently blotted and their fresh weight was recorded, the same were dried in a hot air oven at 90^oc for 48 hours to obtain constant dry weights.

Chlorophyll Estimation:

Through the Arnon method (1949) the total chlorophyll content was estimated .0.2 grams of leaf material was cut into small pieces and blended with 10 ml of 80% acetone in a clean mortar. The green slurry was centrifuged at 3000 rpm for 12 minutes. The supernatant was transferred into a clean test tube the residual pigment in the pellet is re-extracted with 10 ml acetone. The process is repeated till a complete white pellet is obtained. The total volume is made up to 25 ml with 80% acetone. The optical density was determined at 663&645 using 80% acetone solvent as blank in a spectro photometer.

$$\text{Total Chlorophylls} = (0.D\ 645 \times 20.2) + (0.D\ 663 \times 8.02) \text{ V}/1000 \times W$$

$$\text{Chlorophyll a} = (0.D\ 663 \times 12.7) - (0.D\ 645 \times 2.69) \text{ V}/1000 \times W$$

$$\text{Chlorophyll b} = (0.D\ 645 \times 22.9) - (0.D\ 663 \times 4.68) \text{ V}/1000 \times W$$

Assay of Lipid Peroxidation:

Lipid peroxidation in roots of 8 days old Wheat seedlings were determined by estimating the malondialdehyde content according to the method of Stewart & Bewley 1980.

0.2gram of root samples was homogenized in 5 ml of double distilled water. An equal volume of 0.5% Thiobarbituric acid (TBA) in 20% Trichloroacetic acid solution was added and the sample incubated at 95^oC for 32 minutes. The reaction was stopped by putting the reaction tubes in the ice bath. The samples were then centrifuged at 18,000 rpm for 32 minutes. The supernatant removed, absorption was read at 532 nm, and the amount of nonspecific absorption was read at 600 nm and subtracted from this value. The amount of MDA present calculated from the extinction coefficient of 155 mM⁻¹cm⁻¹. Enzyme activity and MDA content of samples were recorded in triplication, and expressed as nM/g.fr.wt.

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 8, August 2016

$$\text{MDA (nM g-1fr.wt.)} = [(A_{532} - A_{600}) \times V \times 1000 / \epsilon] \times W.$$

Where ϵ is the specific extinction coefficient (155mM Cm-1), V is the volume of crushing medium, W is the fresh weight of root, A600 is the absorbance at 600 nm wave length and A532 is the absorbance at 532 nm wave length.

III. RESULTS AND DISCUSSION

Fig..a,b,c,d Shows delayed germination of Wheat seedlings with Al treatment when compared to control. Table No.1 shown that the high concentration of Al considerably decreased the root and shoot growth. Decreasing of total growth of Wheat seedlings was observed as with increasing Al concentrations. Particularly the growth inhibition was clearly seen at 7.5mM and 9.5mM concentrations. Control plants which were pretreated with double distilled water were larger in size with higher seedling mass when compared to treated plants.

In the present study, root and shoot growth of Wheat plant was studied with Al treatment. Root growth was more affected with Al treatment than shoot growth. The same symptom was observed by Petterson and Strid (1989) in Wheat. The inhibition of root elongation was the first visible symptom of Al toxicity and the effect on shoot was the delayed and indirect response to Al^{+3} toxicity. (Fageria 1985; Narayana and Syamala 1989).

The symptoms of toxicity appeared with reduction in seedling growth.

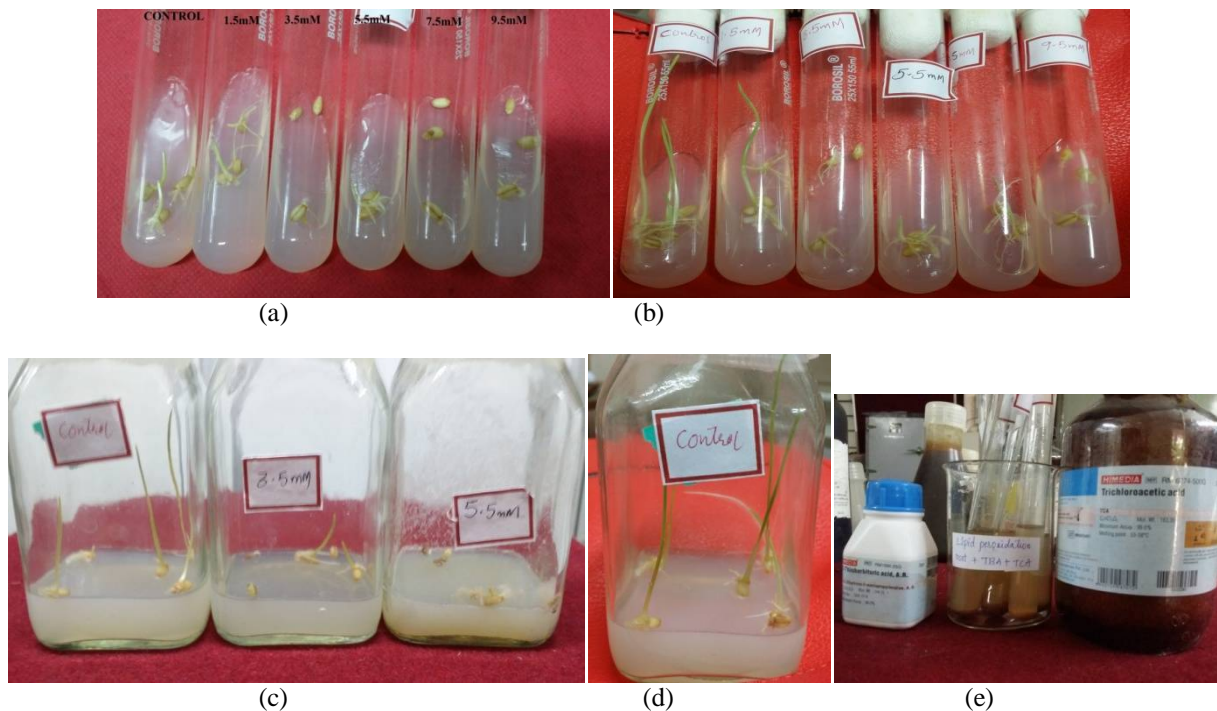


Fig No 1: Effect of Al on *in vitro* Wheat seedlings (a) 5th day (b) 8th day (c) 5th day of Wheat seedlings under different concentrations of Al (d) Control Wheat seedlings 8th day (e) Root lipid peroxidation of Wheat seedlings.

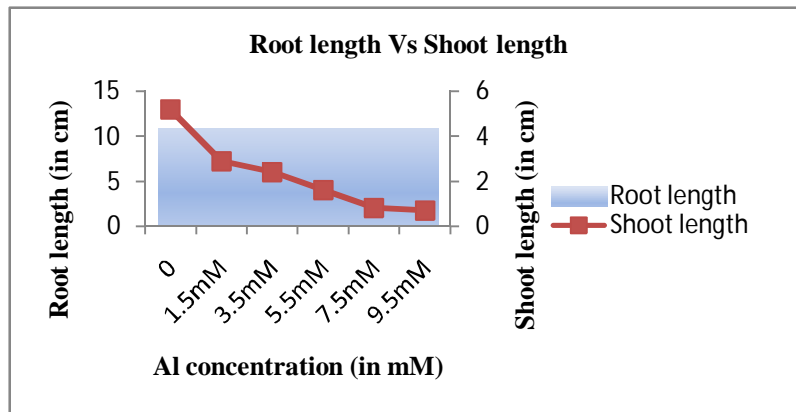
International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

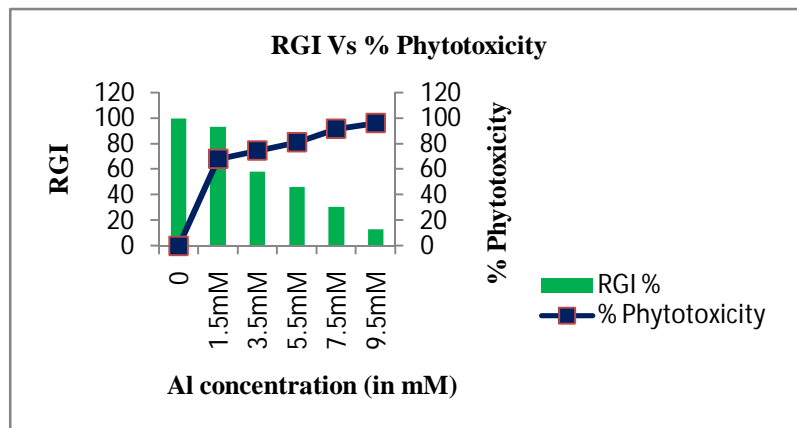
Vol. 5, Issue 8, August 2016

Table No 1: Effect of Al on root length, shoot length, fresh weight and dry weight, RGI and % Phytotoxicity of *in vitro* Wheat seedlings.

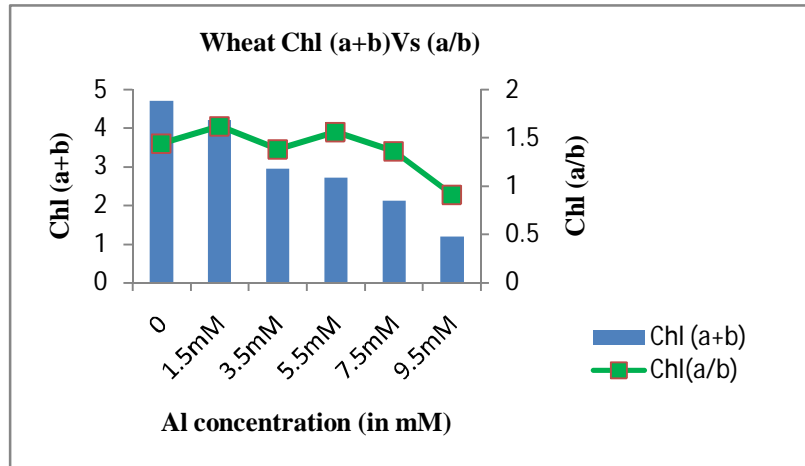
Al conc. (in mM)	Root length (in cm)	Shoot length (in cm)	Fresh weight(in mg)	Dry weight (in mg)	RGI %	% Phytotoxicity
0	10.7	5.2	5.01	3.12	100	0
1.5mM	3.4	2.9	4.14	2.91	93.26	68.22
3.5mM	2.7	2.4	2.96	1.82	58.33	74.76
5.5mM	2.0	1.6	2.32	1.44	46.15	81.30
7.5mM	0.9	0.8	1.91	0.96	30.76	91.58
9.5mM	0.4	0.7	0.92	0.41	12.82	96.26



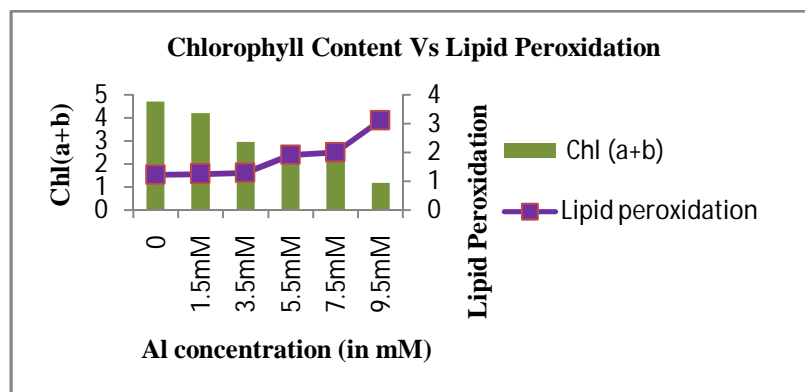
(a)



(b)



(c)



(d)

Fig No 2: (a) Shoot length and Root length decreased with increase in Al concentration in Wheat seedlings. (b) Relative Growth Index (RGI) decreased with increase in % Phytotoxicity in Wheat seedlings (*in vitro*) (c) Total chlorophyll content and chlorophyll a/b ratio decreased with increase in Al concentration in Wheat seedlings. (d) Total chlorophyll content decreased with increase in Lipid peroxidation in Wheat seedlings.

RGI and % Phytotoxicity:

As Al concentration was increased, the % survival was decreased in the culture. The reduction in the amount was noted in fresh weight and dry weight of Wheat seedlings which was significant with the increase of Al treatment. Additionally, the relative growth index was also decreased with increased Al concentrations. At concentrations of 1.5mM of Al the RGI was 93.26% and it was 12.82% at 9.5 mM was noted (Table No:1 and Fig No:2b). Dry matter content was also decreased significantly in response to higher Al concentrations. This was noted with contrast to increasing % of phytotoxicity. At a concentrations of 1.5mM of Al, the % phytotoxicity 68.22% and it was 96.26% at 9.5mM. The present study results indicate that there is a linear relationship between % of phytotoxicity and Al concentration treatments in Wheat seedlings. Particularly, the maximum phytotoxicity symptoms were observed at 9.5mM Al concentration treatment in the present study. Similar trend in % phytotoxicity as a result of Al treatment was observed in Wheat, Barely. The % survival and growth of the plant retarded at higher concentrations of lead by Ashwini A, Waoo, Swati Khare, Sujatha ganguly.

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 8, August 2016

Chlorophyll content:

Results from the measurements of chl a, chl b total chlorophyll content and chl a/b ratio are shown in Table No:2 and Fig No:2c .These results indicate that there were significant decrease in the content of chl a, chl b and total chlorophyll when compared to the control seedlings. The decline in total chlorophyll content and the growth inhibition can be regarded as general responses associated with the Al toxicity. (Rout *et al.*, 2001; Artetxe *et al.*, 2002; rout and Das, 2003). Similar results were observed by Sar kunan *et al.*, 1984, Fageria et al 1988.

Table No 2: Chl a, Chl b, total chlorophyll content and chlorophyll a/b ratio of Al treated Wheat seedlings.

Wheat				
Metal conc. (in mM)	Chl a	Chl b	Chl (a+b)	Chl(a/b)
0	2.78	1.93	4.71	1.44
1.5mM	2.61	1.61	4.22	1.62
3.5mM	1.72	1.24	2.96	1.38
5.5mM	1.66	1.06	2.72	1.56
7.5mM	1.21	0.92	2.13	1.31
9.5mM	0.62	0.52	1.20	1.30

Lipid Peroxidation :

As shown in Table No:3 and Fig No: 1e, 2d. The MDA content increased significantly in Al treated Wheat seedlings under Al stress compared with control plants. The amount of MDA in Al stressed seedlings was increased in all Al treated seedlings. It seems that Al toxicity increased the accumulation of lipid peroxidation product, MDA, which is regarded as an indicator of the loss of structural integrity in membranes subjected to heavy metal stress. (Posmyk *et al.*, 2009; Hosseini *et al.*, 2014).

Oxidative damage is characterized by increasing the levels of the oxidative products such as thiobarbituric acid reactive substances (TBARS), and protein carbonyls. As a result of lipid peroxidation a great variety of aldehydes can be produced, including hexanal, malondialdehyde (MDA) (Catala, 2006).

Oxidative stress is a well known mechanism of cellular injury that occurs with increased lipoperoxidation of cell phospholipids and that has been implicated in various cells dysfunctions (Sies, 1991a, b; Catala, 2006).

Table No 3: Effect of Al on Root lipid peroxidation of Wheat Seedlings.

Metal conc. (in Mm)	Wheat Root Lipid peroxidation
0	1.234
1.5mM	1.264
3.5mM	1.299
5.5mM	1.926
7.5mM	2.012
9.5mM	3.121

IV. CONCLUSION

All the factors affecting by the Al treatment contribute to the inhibition of Wheat plant growth and eventually affect the yield of this important plant crop. Hence, it is necessary to understand the role and effect of heavy metal stress on

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 8, August 2016

plants growth, physiology and metabolism. The present study can further add awareness about the Al toxicity on Wheat plants seedlings growth and physiology.

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International Journal of Innovative Research in Science, Engineering and Technology

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Vol. 5, Issue 8, August 2016

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