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Growth and Physiology of *in Vitro* Planted Seedlings of Flax (*Linum usitatissimum*) Under Aluminum Stress

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Abstract: ***Aluminum stress is one of the key factors for crop yield along with other abiotic stresses such as drought, salinity, extreme temperature and light in acidic soils. Due to natural and anthropogenic activities heavy metal pollution is increasing daily. Hence it is must to know the effects of heavy metals in depth on crop growth and development ultimately yield levels. The main aim of this work was to examine the toxic effect of Aluminum under in vitro growth conditions. Our results clearly demonstrated that Al reduces root growth, shoot growth, fresh weight, dry weight and total chlorophyll content of Flax seedlings. MDA contents in tissues were assayed. The results suggested that the maximum growth inhibition was at higher concentrations of Al. In this experiment with increasing Al treatment resulted in a significant increase in MDA level, an indicator of lipid peroxidation.***

Keywords: *In vitro*, RGI (relative growth index), % phytotoxicity, MDA, lipid peroxidation

1. Introduction

Acid soils are phytotoxic as a result of nutritional disorders, deficiencies of essential nutrients such as Calcium, Magnesium, Molybdenum and Phosphorus and toxicity of Al, Mn and hydrogen activity (Foy *et al.,* 1978; Foy, 1984; Carver and Own by 1995; Jayasundara *et al.,* 1998).

In acid sulfate soils, the very high acidity, with a pH sometimes below 2, poses stress with all the adverse effects of extreme acidity. Furthermore the high Al content of the soil solution has an intensive toxic effect. Soils contain an average of 7% the total Aluminum and under acidic conditions, Al is solubilized, increasing availability to plants and aquatic animal's soil acidification due to application of fertilizers growing of legumes or acid rain is an increasing problem in agricultural and natural ecosystems. No conclusive evidence suggests that Aluminum is an essential nutrient for either plants or animals. Al is toxic to plants and animals, interfering with cytoskeleton structure and function, disrupting calcium homeostasis interfering with phosphorus metabolism and causing oxidative stress. 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 phytoremediation and improved tolerance, it is important to understand as to how heavy metals are taken up and act at cellular and tissue level (Schutzendubel and Polle, 2002; Beak *et al.,* 2006).

Flax (also known as common Flax or Linseed or Ullusallu or Madanaginja), **(*Linum usitatissimum,*** is a member of the genus Linum in the family *Linaceae.* It is a food and fiber craft cultivated in cooler regions of the world. Basically this crop thrives well in alluvial soil. This crop requires a relative humidity of 50 - 60%, along with seven inches (7") of rain and with a pH of 6.0 – 6.5. Major Flax seed grown states in India were Madhya Pradesh, Uttar Pradesh, Bihar, Chhattisgarh, Maharashtra, Jharkhand, Odessa, Assam, Nagaland, West Bengal, Himachal Pradesh, Rajasthan, Karnataka, Andhra Pradesh and Telangana.

Medicinal uses:
Flax flower and seed oil used for diabetes, swellings, abscess, eye diseases, skin diseases, diarrhea, constipation, leucorrhoea, cough, micturition, convulsions , hemiplegia , sprue, cardiac diseases, odema, dysponoea, febrifuge. The main aim of the research programme is to focus on the mechanisms of Al toxicity with an emphasis on the plant physiological response of the Flax seedlings to Aluminum stress. The different concentration of Al (1.5mM, 3.5mM, 5.5mM, 7.5mM and 9.5mM) on seedling growth, chlorophyll content and lipid peroxidation in the Flax seedlings analyzed to understand the physiological response of Flax seedlings under Al stress in *in vitro* growth conditions.

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:

1) 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_4NO_3	16.5	100.0
3.	KH_2PO_4	1.7	100.0
4.	$MgSO_4.7H_2O$	3.7	100.0

The above mentioned salts were taken into a 2 liter beaker, dissolved initially in 500ml distilled water (H_20). Finally the volume was made up to1000ml by adding distilled water

.Then this solution was labeled as stock- I solutions and stored at 4^0c.

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

S. No.	Nutrient Name	Quantity in g (10 × 100)	Volume of Stock (in ml/L)
1.	$CaCl_2 .2H_2O$	4.4	100.0

4.4g of $CaCl_2.2H_20$ 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 (H_20). Stored at 4^0c. This is the Stock – II.

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

S. No.	Nutrient Name	Quantity in g (10 × 100)	Volume of Stock (in ml/L)
1.	H_3BO_3	62	100.0
2.	$MnSO_4. H_2O$	168.9	100.0
3.	$ZNSO_4.7H_2O$	86	100.0
4.	KI	8.3	100.0
5.	$Na_2MoO_4.2H_2O$*	2.5	100.0
6.	$CuSO_4.5H_2O$**	0.25	100.0
7.	$Cocl_2.6H_2O$**	0.25	100.0

*Prepared separately and then added
**Prepared 100 mg in 100 ml DDH_2O 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 H_20 and finally made up the volume up to 1000ml by adding distilled H_20 and labeled it has stock- III solution and Stored at 4^0c.

4) 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. $2H_2O$	3.73	100
2	$FeSO_4$. $7H_2O$	2.78	100

1000ml double distilled water was taken in a 1500 ml of amber colored bottle and warmed the water up to near boiling. Then added Na_2 EDTA. $2H_2O$ while stirring under magnetic stirrer. After it get dissolved added $FeSO_4$ 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 4^0C.

5) 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 4^0C.

6) 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
2	MS-II	25
3	MS-III	25
4	MS-IV	25
5	MS-V	25
6	Sucrose (gm)	30
7	Calciumdpentatinate	2

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 $Hgcl_2$ 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 25^0c, 70% RH and irradiance of 50-60 µmol $m^{-}2$ s^{-1}T 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 8^{th} 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:

Percent phytotoxicity = root length of control—root length of test ×100

Root length of control

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 90^0c 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.

Total Chlorophylls = (0.D 645 × 20.2) + (0.D 663 × 8.02) V/1000× W

Chlorophyll a = (0.D 663 × 12.7) -- (0.D 645 × 2.69) V/1000× W

Chlorophyll b = (0.D 645 × 22.9) -- (0.D 663 × 4.68) V/1000× W

Assay of Lipid Peroxidation:

Lipid peroxidaion in roots of 8 days old Flax seedlings were determined by estimating the melondialdehyde content according to the method of Stewart & Bewley 1980.

0.2 gram 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°C 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/gr.fr.wt.

MDA (nM gr-1fr.wt.) = [(A532 – A600) × V× 1000/ ε] × 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.

2. Results and Discussion

Al toxicity causes significant changes in Flax seedling growth. Young seedlings are more susceptible than older plants. Normally the root is most affected by Al toxicity as a result root elongation is considered to be the most sensitive parameter in a short period of time. Aluminum does not interfere with seed germination but does impair the growth of new roots and seedling establishment (Nosko *et al.,* 1988).

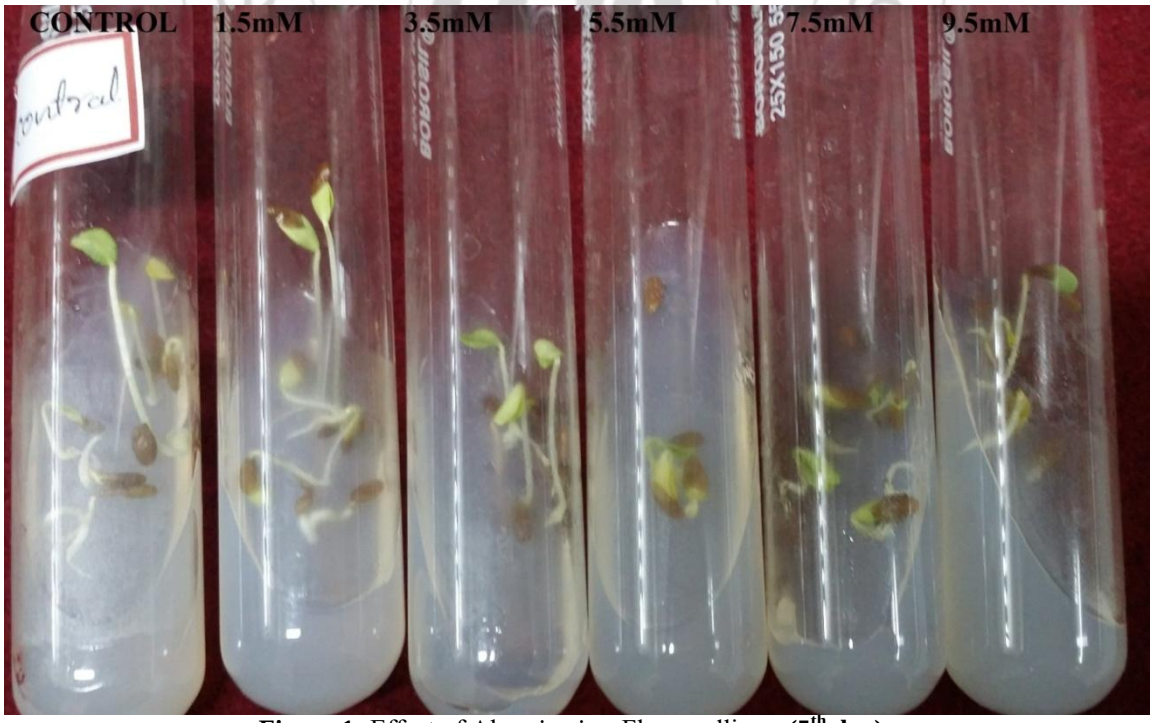

Figure 1: Effect of Al on *in vitro* Flax seedlings. **(5th day).**

Figure 1(a): Effect of Al on *in vitro* Flax seedlings. **(7th day).**

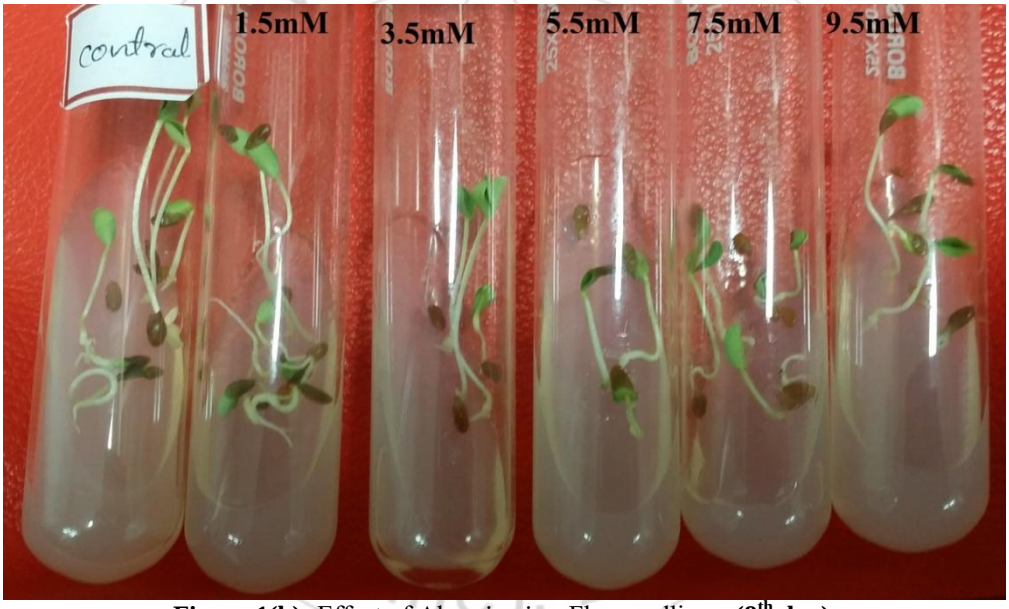

Figure 1(b): Effect of Al on *in vitro* Flax seedlings. **(8th day)**

Table 1: Effect of Al on root length, shoot length, fresh weight and dry weight, RGI and % Phytotoxicity of *in vitro* Flax seedlings

Al conc. (in mM)	Root length (in cm)	Shoot length (in cm)	Fresh weight (in mg)	Dry weight (in mg)	RGI %	% Phytotoxicity
0	3.2	5.1	4.52	2.87	100	0
1.5mM	2.6	4.9	3.41	2.12	73.86	18.75
3.5mM	2.0	3.8	3.05	2.02	70.38	37.50
5.5mM	1.2	3.0	2.14	1.37	47.73	62.50
7.5mM	0.8	2.7	1.72	0.87	30.31	75.00
9.5mM	0.2	1.8	1.26	0.42	14.63	93.75

Fig 1a, 1b, 1c, 2 and Table 1, shows that increasing the concentrations of Aluminum from 1.5mm to 9.5mM progressively decreased root growth and reduction in shoot length was also observed. At higher concentrations of Aluminum the root growth was more affected than the shoot growth and as observed by Pettersson 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).

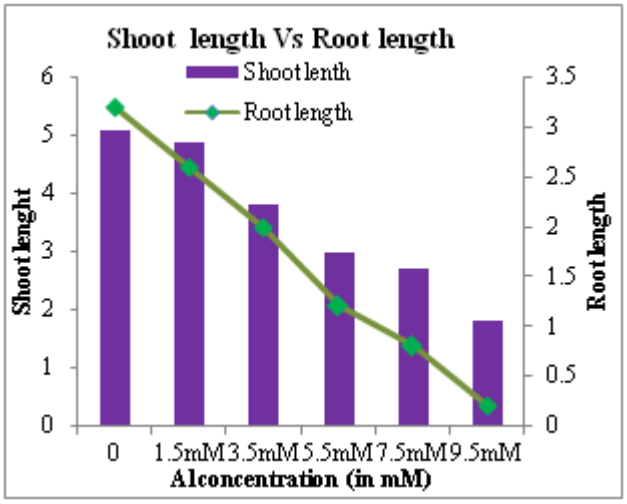

Figure 2: Shoot length and Root length decreased with increase in Al concentration in Flax seedlings.

% Phytotoxicity :

The effect of Al on % phytotoxicity and RGI of Flax seedlings is depicted in Fig no 2 and Table no 1. In 8th day old seedlings % phytotoxicity showed a linear decrease with increase in Al treatment. The recorded % phytotoxicity was 18.75% at 1.5mM of Al treatment and significantly \increased to 93.75% at 9.5mM. Similar trend in %phytotoxicity as a result of Al treatment was observed in Wheat seedlings (Saritha *et al.*, 2016).

RGI:

The RGI was calculated from the dry weight of roots and shoots of Flax seedlings is depicted in Table no 1 and Fig No 2a. The RGI of roots and shoots of Flax seedlings decreased with increasing Al treatment. At 1.5mM the RGI was 73.86% at 9.5mM the RGI was 14.63% (85.37% reduction). Similar results were obtained for Zn and Ni treated pigeon pea cultivars (Sresty *et al.*, 2000). In the present study dry matter content significantly decreased in response to higher Al concentrations. In contrast increasing Al concentrations increased % phytotoxicity in Flax seedlings (**Fig No:** 2a).

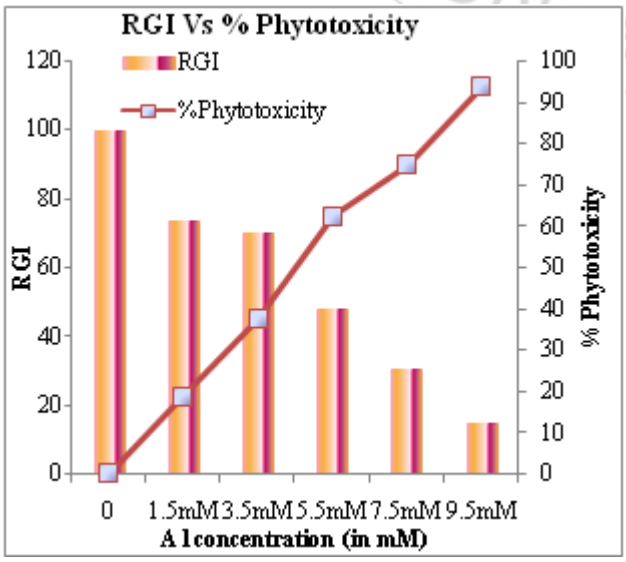

Figure 2(a): Relative Growth Index (RGI) decreased with increase in % Phytotoxicity in Flax seedlings (*in vitro*)

Chlorophyll content

Fig No 2b and Table No 2 shows chlorophyll a, chlorophyll b and the total chlorophyll contents as affected by Aluminum. Chl a, Chl b and total chlorophyll content and ratio were decreased significantly with increasing Al concentrations. The maximum decrease of chlorophyll content was observed at 9.5mM Al. which indicates that Al inhibits chlorophyll biosynthetic path way enzymes such as δ- amino levulinic acid dehydrogenase and protochlorophyllide reductase.

These results indicate that there were significant decreases in compared with 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 Sarkunan *et al.*,1984, Fageria *et al.*, 1988.

Table 2: Chl a, Chl b, total chlorophyll content and chlorophyll a/b ratio of Al treated Flax seedlings

Flax			
Chl a	**Chl b**	**Chl (a+b)**	**Chl(a/b)**
3.34	2.01	5.35	1.66
2.79	1.87	4.66	1.49
2.12	1.56	3.68	1.35
1.79	1.25	3.04	1.43
1.36	1.04	2.40	1.30
0.91	0.57	1.48	1.59

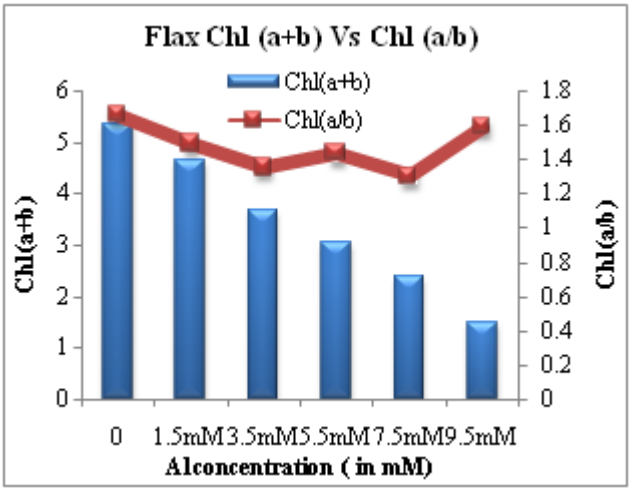

Figure 2b: Total chlorophyll content and chlorophyll a/b ratio decreased with increase in Al concentration in Flax seedlings

Lipid Peroxidation

Fig No: 2c and Table No 3 demonstrates that Al treatment resulted in a significant increase in MDA content as an indicator of lipid peroxidation .This indicates that Al induced oxidative damage as linked to lipid peroxidation in Flax roots. The metal induced lipid peroxidation is mostly attributed to increased production of ROS(reactive oxygen species) especially OH radicals in plant systems (Halliwell and Gutteridge, 1984). Malondialdehyde is considered to be the general indicator of lipid peroxidation, which can be started by the redox –active metal ions themselves. This phenomenon can be imitated by the iron containg enzyme lipoxygenase (Thompson *et al.*, 1987). This membrane bond

enzyme, which is known to produce pre radicals, may be responsible for increase in MDA level on Cd treatment (Somashekaraiah *et al.,* 1992).

Our results indicate that excess Al induced oxidative damage, as is evident from increased lipid peroxidation in roots of Flax seedlings. Similar results were observed in other plants for various heavy metals (Lin and Koo, 2001; Wang at el., 2004). Increasing lipid peroxidation suggests an Al mediated membrane disruption in plant cells (Wang *et al.,* 2004). Yamamoto *et al.,* (2001) have shown that peroxidation of lipids is a relatively early event following Al exposure. Malondialdehyde is considered to be the general indicator of lipid peroxidation.

Table 3: Effect of Al on Root lipid peroxidation of Flax Seedlings

Metal conc. (in Mm)	*Flax Root Lipid peroxidation*
0	1.026
1.5mM	1.248
3.5mM	1.271
5.5mM	1.187
7.5mM	1.992
9.5mM	2.062

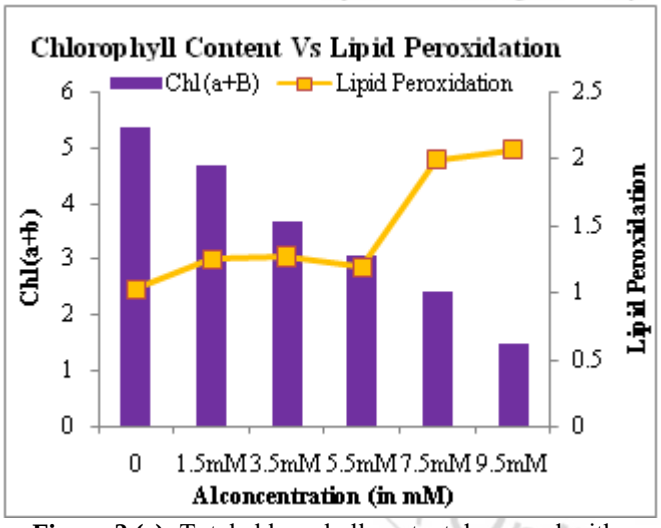

Figure 2 (c): Total chlorophyll content decreased with increase in Lipid peroxidation in Flax seedlings

3. Conclusions

The results demonstrated that the Flax seedlings were very sensitive to Al application *in vitro*. Al induced reduction of root growth, shoot growth, chlorophyll content and enhancement of lipid peroxidation in Flax seedlings could be observed. However, germination rates of seeds were not affected, but delayed with Al treatment. All these factors contributed to the inhibition of plant growth and could affect these important crop plants. It is thus necessary to understand heavy metals stress induced response in these highly valued edible plants to ensure a huge quality product for the consumer.

4. Acknowledgements

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