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EFFECT OF ALUMINUM ON *IN VITRO* GROWTH AND PHYSIOLOGY OF WHEAT AND FLAX SEEDLINGS

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Abstract

This research was designed to study the Aluminum stress on Wheat and Flax seedlings in culture. The cultures of Wheat and Flax were firstly established on MS Media supplemented with Aluminum, BAP, IAA and the stable *in vitro* plantlets then used for further study. The effect of Al on Wheat and Flax seedlings under various concentrations were studied using invitro culture. The root growth, shoot growth, fresh weight, dry weight, RGI (relative growth index), % phytotoxicity and MDA contents in tissues were assayed. The results suggested that the maximum growth inhibition was at higher concentrations of Al. Al treatment resulted in a significant increase in MDA level, an indicator of lipid peroxidation.

Key words: *In vitro*, BAP, IAA, RGI (relative growth index), % phytotoxicity, MDA, lipid peroxidation.

Introduction

Most heavy metals function as a toxic pollutant for human, animals and plants, which enters the environment mainly from industrial processes and phosphate fertilizers and then is transferred to the food chain with potential consequences for human health (Jarup, 2003). 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, Jayasundara *et al.,* 1988). Al injured roots are stubby and brittle. Root tips and lateral roots thicken and turn brown the root system as a whole is affected by many stubby lateral roots and no fine branching. Such roots are inefficient in absorbing nutrients and water (Foy *et al.,* 1978). The main symptom of Al toxicity is the rapid inhibition of root growth.

Materials and Methods

MS medium was used as a basal medium in the 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 for time saving purpose.

Composition of MS medium

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

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The above mentioned salts were taken into a 2 liter beaker, dissolved initially in 400ml 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.

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

4.4g of $CaCl_2 2H_20$ was taken into a 2.0 liter beaker and then dissolved with 400ml distilled water and finally made up the volume 1000ml by adding distilled water (H_20). Stored at 4^0C. This is the Stock-II.

III Micro stock (MS-III) in 1000 ml

*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 400ml 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.

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

1000ml double distilled water was taken in a 1000 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 is dissolved added $FeSO_4$ gradually and mild stirring was done using a magnetic stirrer. Then closed the bottle immediately and kept on

Table 1: Stock Solution I.

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

Table 2: Stock Solution II.

S. No.	Nutrient Name	Quantity in g(10 × 100)	Volume of Stock (in ml/L)
1.	$CaCl_3.2H_2O$	4.4	100.0

Table 3: Stock Solution III.

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

Table 4: Stock Solution IV.

S. No.	Nutrient Name	Quantity in g(10 × 100)	Volume of Stock (in ml/L)
1.	$Na_2 2H_2O$	EDTA.	3.73 100.0
2.	$FeSO_4.7H_2O$	2.78	100.0

Table 5: Stock Solution V.

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

Table 6: Stock Solution VI.

S. No.	Stock	Quantity in ml or g per liter
1.	MS-I (40X)	25.0
2.	MS-II (40X)	25.0
3.	MS-III (40X)	25.0
4.	MS-IV (40X)	25.0
5.	MS-V (40X)	25.0
6.	Sucrose (gm)	20.0
7.	Calciumdpentatinate	2.00

stirring for an hour. And then labeled it as MS-IV and stored at 4^0C.

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

400 ml double distilled water was taken in a 2 liter beaker and then added every salt sequentially in 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.

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.

Preparation of 1 liter of MS basal Medium

For the preparation of 1 liter of MS Basal Medium, all the above mentioned stock solutions were added sequentially in about 400 ml of double distilled water. And then added 20 g of sucrose and dissolved it with the help of magnetic stirrer.

Then IAA and 6-BAP the medium growth regulators were added and the volume of the medium was made up to 1000 ml by adding distilled water. The 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 were used (i.e., 8.0g, 9.0g, 11g, 14g and 22g), because the increasing concentration of Al may interfere solidification of the medium. The 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 is 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 airflow. 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 24^0C, 70% RH and irradiance of 40-60 µmol m^2 s^{-1}T with 16 hours photoperiod. All the aseptic

conditions were maintained throughout the period of seed germination.

Growth conditions

Seeds were surface sterilized with 0.001M mercuric chloride for two minutes and thoroughly washed with water several times. Sterilized seeds were cultured on Ms medium containing different concentrations of $Al_2(SO_4)_3$ (1.5mM, 3.5mM , 5.5mM, 7.5mM, 9.5mM) and solidified with different amounts of agar (9, 10, 13, 18, 22 grams) respectively. Since increasing Aluminum concentrations affected the solidification of media. The pH of media was adjusted to 4.0 before autoclaving. All cultures were maintained in a growth chamber at 24^0C, 70% relative humidity and irradiance of 40-60 µmol $m^{-2}s^{-1}$ T (cool white fluorescent tubes) with 16 hours photoperiod.

Sample collection

The plants were first removed from the culture medium, the entire plant with roots, shoots were put under a constant flow of water to remove the culture medium particles and exogenous contaminants adhered to the plants.

The water droplets were blotted dry with the help of blotting paper. Sampling was done in the early hours for the measurement of various morphological, growth and biochemical parameters.

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 the length of each part was measured using a graph paper.

Percent phytotoxicity:- It was calculated as follows:

Percent phytotoxicity =

$$\frac{root\ lenght\ of\ control - root\ length\ of\ test \times 100}{Root\ length\ of\ control}$$

Dry weight:- The seedlings were separated into roots and shoots, gently blotted and their fresh weight was recorded, the same was dried in a hot air oven at 90^0C for 48 hours to obtain constant dry weights.

Assay of Lipid Peroxidation

Lipid peroxidation in roots of 8 days old Wheat and Flax seedlings were determined by estimating the malondialdehyde 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 $mM^{-1}cm^{-1}$ Enzyme activity and MDA content of samples was 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 wavelength and A532 is the absorbance at 532 nm wavelength.

Results and Discussion

Table 7 and Fig. 1 shows that the increased Al concentration considerably decreased the root growth, shoot growth decreased as the Al concentration increases the total growth of Wheat and Flax seedlings reduces. Control plants pretreated with double distilled water was

Table 7: Effect of Al on root length, shoot length, fresh weight and dry weight, RGI and % Phytotoxicity of invitro Flax seedlings.

Al conc. (in mM)	Root length (in cm)	Shoot length (in cm)	Fresh weight (in mg)	Dry weight (in mg)	RGI %	% Phytot-oxicity
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

Table 8: Effect of Al on root length, shoot length, fresh weight and dry weight, RGI and % Phytotoxicity of invitro Wheat seedlings.

Al conc. (in mM)	Root length (in cm)	Shoot length (in cm)	Fresh weight (in mg)	Dry weight (in mg)	RGI %	% Phytot-oxicity
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

Table 9: Effect of Al on Root lipid peroxidation of Wheat and Flax Seedlings.

Metal conc. (in Mm)	Wheat Root Lipid peroxidation	Flax Root Lipid peroxidation
0	1.234	1.026
1.5mM	1.264	1.248
3.5mM	1.299	1.271
5.5mM	1.926	1.187
7.5mM	2.012	1.992
9.5mM	3.121	2.062

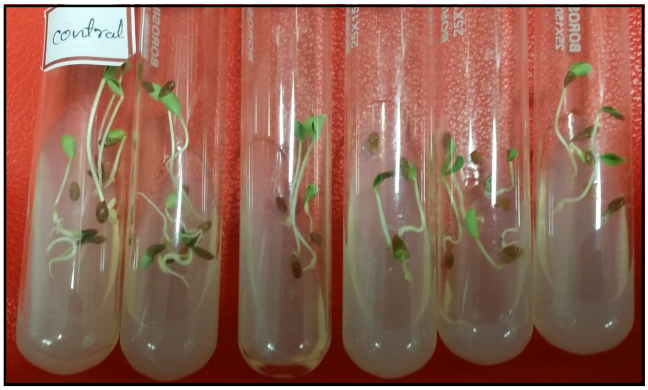

Fig. 1: Effect of Al on invitro Flax seedlings.

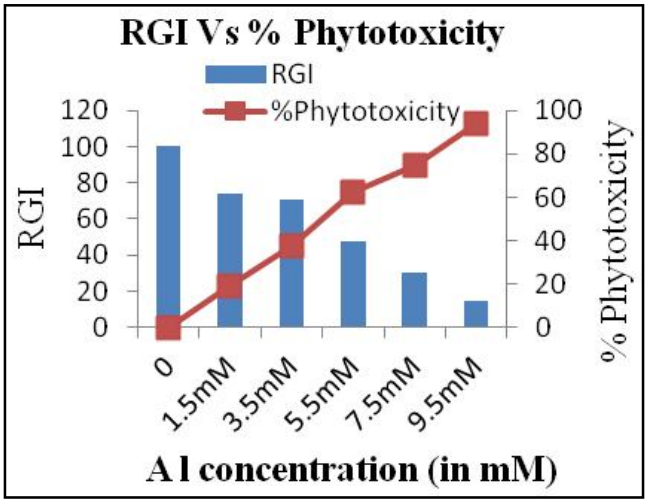

Fig. 2: Relative Growth Index (RGI) decreased with increase in % Phytotoxicity in Flax seedlings (invitro).

considerably larger as indicated by higher seedlings mass. In the present work root growth was affected more than that of the shoot and as 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 the shoot was the delayed and indirect response to Al^{+3} toxicity (Fageria 1985; Narayana and Syamala 1989).

RGI and % Phytotoxicity

In addition, the relative growth rate decreased with increasing Al concentrations in the present study. Dry matter content significantly decreased in response to

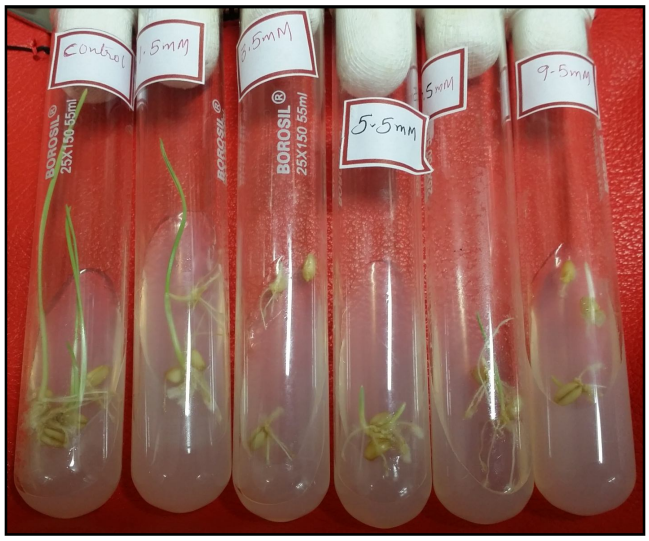

Fig. 3: Effect of Al on invitro Wheat seedlings.

Fig. 4: Control Wheat seedlings.

higher Al concentrations. In contrast increasing Al concentrations increased % phytotoxicity.

Lipid Peroxidation

As shown in Fig. 6 the MDA content increased significantly in Al treated Wheat and Flax seedlings under 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

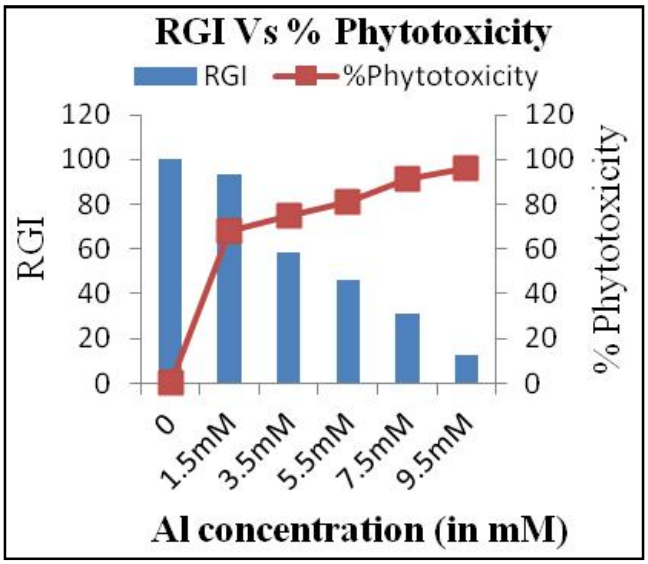

Fig. 5: Relative Growth Index (RGI) decreased with increase in % phytotoxicity in Flax seedlings.

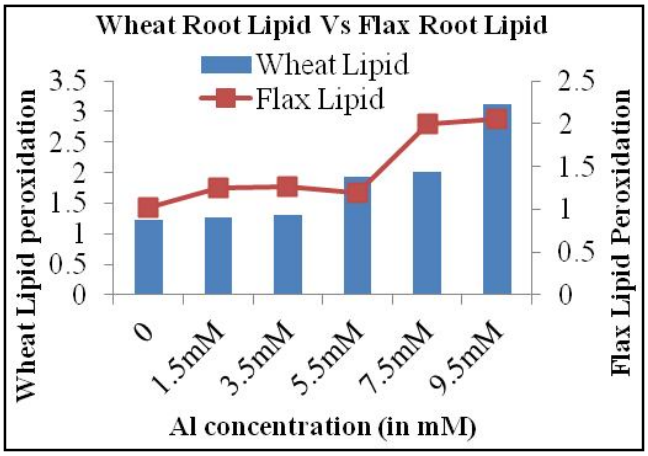

Fig. 6: Root lipid peroxidation increased with increase in Al concentration in Wheat and Flax seedlings.

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).

Conclusions

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.

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