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ISSN: 0976-3031

International Journal of Recent Scientific Research
Vol. 7, Issue, 12, pp. 14571-14575, December, 2016

International Journal of Recent Scientific Research

Research Article

EFFECT OF HEAVY METAL TOXICITY ON *IN VITRO* POLLEN GERMINATION AND TUBE GROWTH OF *PHYLLANTHUS RETICULATUS* POIR.

Megha Dutta Mudi and Subrata Mondal*

Department of Botany, Visva-Bharati, Santiniketan-731235

ARTICLE INFO

Article History:

Received 15th September, 2016
Received in revised form 25th October, 2016
Accepted 23rd November, 2016
Published online 28th December, 2016

Key Words:

Pollen germination, Heavy metals, $CoCl_2$, $Pb(NO_3)_2$, $CdCl_2$, *Phyllanthus reticulatus.*

ABSTRACT

Although some heavy metals are essential trace elements, most of them can be toxic to all forms of life at high concentrations due to formation of complex compounds within the cell. The present study deals with the effect of heavy metals like Cadmium, Cobalt and Lead on *in vitro* pollen germination and tube growth of *Phyllanthus reticulatus* Poir., a medicinally important plant belonging to the family Euphorbiaceae. The study reveals that, heavy metals led to a significant decrease in pollen germination as well as tube growth. Maximum 98% pollen germination along with 884µm pollen tube development was observed in 10% sucrose solution supplemented with 50 ppm boric acid. Reduction in pollen germination and tube elongation was noticed as metal concentrations increased. In 150 µM $CoCl_2$ and $Pb(NO_3)_2$ solution 20% and 7% germination were observed along with 104 µm and 91µm pollen tube respectively. The highest toxicity was found in $CdCl_2$ solution where only 3 % pollen grains along with only bulging out of pollen tubes were found in 150 µM solution. Pollen germination and tube growth were adversely affected by Co^{2+}, Pb^{2+} and Cd^{2+} , thus heavy metals inhibit *in vitro* pollen germination and tube growth which ultimately affect the pollen viability as well as plant sexual reproduction.

INTRODUCTION

A toxic heavy metal is any relatively dense metal or metalloid that is noted for its potential toxicity, especially in environmental contexts. Heavy metals are found naturally on the earth. In recent years, there has been an increasing ecological and global public health concern associated with environmental contamination by these metals. An exponential increase of their use in several industrial, agricultural, domestic and technological applications have risen dramatically (Bradl, 2002). Then, they can bind to interfere with the functioning of vital cellular components. Common sources are from industrial wastes, vehicle emissions, lead-acid batteries, fertilisers (Harvey *et al.,* 2015). Among them, Cadmium has attracted the most attention due to its potential toxicity to man and relatively high mobility into the plant system (McLaughlin and Singh, 1999). Its widespread distribution and high toxicity make it a potential contaminant and a threat to a wide number of natural environments and biota (Traina, 1999). Pollutants of this kind at ppm levels usually cause anomalies in mitotic division and chromosome aberrations (Tuna *et al*. 2002; Njagi and Gopalan, 1981).The permeability of the cell membrane, the biochemical activities at the macromolecular level and the regular growth and reproduction of cells are negatively affected (Aggrawal, 2014). Motor vehicle emissions are a major source of airborne contaminants including arsenic, cadmium, cobalt, nickel, lead, antimony, vanadium, zinc, platinum, palladium and rhodium (Balasubramanian *et al*., 2009). Water sources can be polluted by heavy metals leaching from industrial and consumer wastes, acid rain can exacerbate this process by releasing heavy metals trapped in soil (Worsztynowicz and Mill, 1995). During the past two decades, pollen tubes of various plant species have been used to determine the cytotoxic effect of environmental pollutants (Gentile *et al*., 1973; Masaru *et al*., 1980; Pfahler, 1981; Xiong and Peng, 2001; Gur and Topdemir, 2005).Heavy metals such as Cd, Co, Fe, Pb, Zn, Cu and Hg at toxic levels inhibit pollen germination, pollen tube growth and seed germination, causing ultra-structural changes. Other negatively affected characteristics include germination percentage, germination index, root and shoot lengths, it's dry matter rates etc., (Ayaz and Kadioglu, 1997). Some studies claim that heavy metals, particularly Pb and Cd, in the exhaust gases, engine oils and tires of vehicles in regions with heavy traffic (Krishnayya and Bedi, 1986) and acid rain (Cox, 1984) inhibit pollen germination and tube growth in plants, reaching toxic levels in the plant leaves. Symptoms and effects can vary according to the metal or metal compound and the doses are involved. There are also findings indicating that, heavy metals cause cytogenetic anomalies in plants, like the inhibition of mitosis, decrease in the mitotic index and chromosomal anomalies (Inceer and Beyazoglu, 2000). In biological systems, heavy

*Corresponding author: **Subrata Mondal**
Department of Botany, Visva-Bharati, Santiniketan-731235

metals have been reported to affect cellular organelles and components such as cell membrane, mitochondria, lysosome, endoplasmic reticulum, nuclei and some enzymes involved in metabolism (Wang, 2001). Metal ions have been found to interact with cell components such as DNA and nuclear proteins, causing DNA damage and conformational changes that may lead to cell cycle modulation, carcinogenesis or apoptosis (Chang, 1996). In the present investigation an attempt has been made to study the effects of the heavy metals like $CdCl_2$, $CoCl_2$, and $Pb(NO_3)_2$ on *in vitro* pollen germination and pollen tube growth of *Phyllanthus reticulatus* Poir. as pollen viability in relation to its germination ability has a paramount importance in breeding programme and plant reproduction.

MATERIALS AND METHODS

Standard solutions of each heavy metal under consideration (Co, Pb, Cd) were prepared like 30, 60, 90,120,150 µM of Co as $CoCl_2$, Pb as $Pb(NO_3)_2$ and Cd as $CdCl_2$. Newly opened flowers were collected in the morning (09.30 hrs.-10.30 hrs.) and transferred to polythene bag. The fresh pollen samples were sown on several grooved slides containing solution of sucrose, boric acid, $CoCl_2$, $Pb(NO_3)_2$ and $CdCl_2$. In the experiment, a germination medium containing 10% sucrose with 50ppm H_3BO_3, was used for the germination as basic medium where pollen grains showed maximum 98% germination along with 884µm pollen tube development (Fig:1A,1B; Dutta Mudi and Mondal, 2014). Then different concentration of heavy metals were added with the basic solution to study their effects. Slides were then kept in Petridishes lined with moist filter paper and examined under the microscope at different time intervals to record the germination percentage and pollen tube growth following the method of Shivanna and Rangaswamy (1993). A pollen grain was considered as germinated, if pollen tube length atleast becomes twice greater than the diameter of the pollen grains (Gupta *et al*., 1989).

RESULTS AND DISCUSSIONS

In this study, pollen grains were left to germinate in culture medium containing cadmium, cobalt and lead. Pollen germination and tube growth rate depended mainly on pollen quality. Pollens which were collected from the newly opened fresh flowers showed the highest germinating ability on basic medium (Fig: 1A,1B),whereas adverse effects were observed with 30, 60, 90,120 and 150 µM of heavy metals when added as salts to the medium. The reduction in pollen germination and tube length were noticed in 30 µM solution in all cases. Beyond that concentration, different anomalies like branching, tip swelling, coiling and tip bursting of pollen tubes were recorded. However, 20 % germination along with 104 µm pollen tube and 7% pollen germination along with 91 µm pollen tube were recorded in 150 µM solution of $CoCl_2$, and $Pb(NO_3)_2$ respectively, whereas 3% pollen grains with only bulging out of pollen tubes were observed in 150 µM $CdCl_2$ solution.Cd^{2+}showed the highest toxicity, while germination and pollen tube growth was less affected by Co^{2+} and Pb^{2+} (Table 1-3, Fig.1C-J) due to different tolerance levels of pollen.

Table-1 Effect of $CoCl_2$ on *In Vitro* Pollen Germination of *Phyllanthus reticulatus* Poir.

Conc. Of $CoCl_2$(µM) with 10%Sucrose+ 50ppm Boric Acid	After 1 Hr.		After 2 Hrs.		After 3 Hrs.	
	Germination (%)	Tube Length (µm)	Germination (%)	Tube Length (µm)	Germination (%)	Tube Length (µm)
30	50	364	55	403	65	416
60	40	247	45	273	50	338
90	30	208	35	234	40	312
120	25	130	30	156	35	182
150	10	84	15	91	20	104

Table-2 Effect of $Pb(NO_3)_2$ on *In Vitro* Pollen Germination of *Phyllanthus reticulatus* Poir.

Conc. Of $Pb(NO_3)_2$(µM) with10%Sucrose +50ppm Boric Acid	After 1 Hr.		After 2 Hrs.		After 3 Hrs.	
	Germination (%)	Tube Length(µm)	Germination (%)	Tube Length(µm)	Germination (%)	Tube Length(µm)
30	40	247	45	286	50	351
60	30	208	35	234	40	260
90	17	117	20	130	25	156
120	10	91	12	104	15	117
150	2	65	5	78	7	91

Table-3 Effect of $CdCl_2$ on *In Vitro* Pollen Germination of *Phyllanthus reticulatus* Poir.

Conc. Of $CdCl_2$ (µM) with 10%Sucrose+ 50ppm Boric Acid	After 1 hr.		After 2 hrs.		After 3 hrs.	
	Germination (%)	Tube length(µm)	Germination (%)	Tube length(µm)	Germination (%)	Tube length(µm)
30	35	78	40	91	45	117
60	25	52	32	65	35	78
90	12	26	15	26	20	39
120	5	Bulging out	8	Bulging out	10	Bulging out
150	-	-	2	Bulging out	3	Bulging out

Affected tubes showed different anomalies like swelling of the tip region, thick tube wall, uneven tube developments, coiling of tubes and tube bursting which may be due to changes involved in cytoskeleton and actin filaments, vesicular structure and cell wall organisation (Sawidis, 2008; Sawidis and Reiss, 1995). Present investigation showed that pollen tube elongation was greatly inhibited by high concentrations of heavy metal solutions. Cadmium reduced the average pollen germination and tube length drastically. The tubes showed an increasing tendency to burst after 3 hrs. of incubation. However, the pollen tube growth pattern differed among them, revealing a great variability in development. These abnormalities led to slowing down or complete cessation of pollen tube elongation.

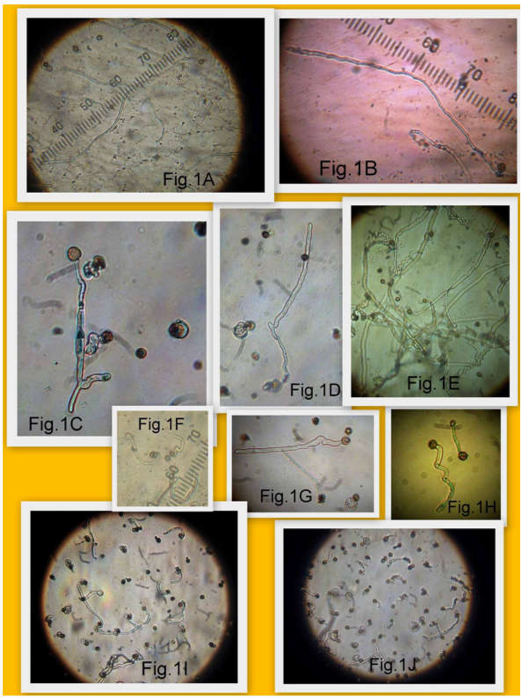

Fig.1A,1B Normal Germinating pollen in basic medium(10%sucrose+50ppm boric acid);Fig.1C,1D Branched pollen tube development in 30 µM $CdCl_2$ solution; Fig.1E Coiling of pollen tubes in 60 µM $Pb(NO_3)_2$ solution ; Fig.1F Swelling of tip region of pollen tubes in 90 µM $CoCl_2$ solution. Fig.1G and H Coiling and uneven pollen tubes in 90 µM $Pb(NO_3)_2$ solution. Fig.1I Reduction of germination percentage and pollen tube length in 120 µM $Pb(NO_3)_2$ solution.Fig.1J Reduction of germination percentage and pollen tube length in 120 µM $CdCl_2$ solution.

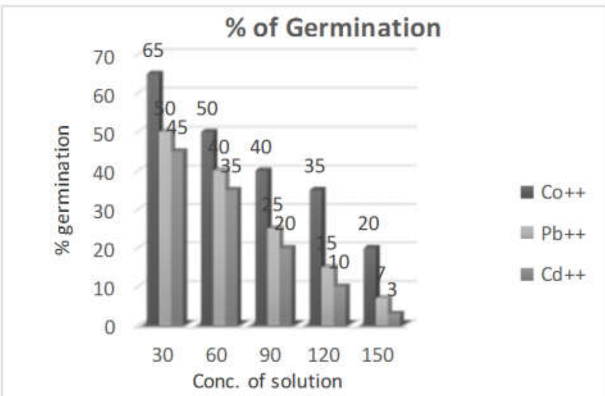

Fig.2A

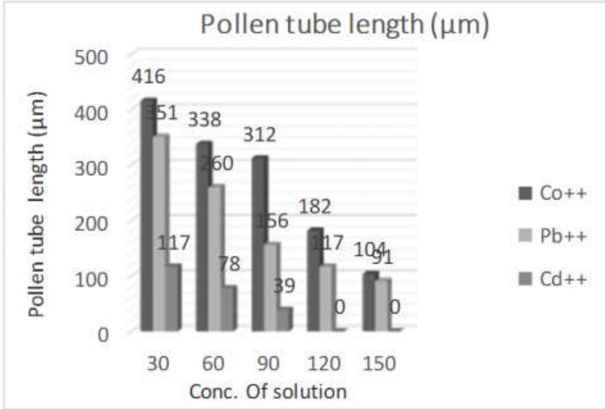

Fig.2B

Fig.2A Showing Pollen germination rate and Fig.2B Showing Pollen tube length in different concentration of heavy metals.

A growing pollen tube is a highly active secreting cell, exhibiting oriented exocytosis at the tube tip, including vesicle transport and membrane fusion. The most obvious feature of a normally growing pollen tube is the accumulation of vesicles at the tip region, causing the formation of the characteristic vesicular zone. Results showed that both pollen germination and pollen tube lengths were negatively affected by the application of heavy metals in increasing concentrations. As the metal increased, the length of the tip region was reduced (Sawidis, 2008). Loosening of the tube wall as well as increase in wall thickness was noticed with the increasing metal concentrations. It has also been claimed that Cd, Cu, Hg and Ni are more toxic than Pb and Zn for plants (Raskin *et al*., 1994). Heavy metals not only affect the electron transportation during respiration negatively but also inhibit the plant growth indirectly by preventing enzyme activity partly or completely (Larcher, 1995). It was observed that the formation of uneven pollen tubes deviating from the normal straight growth would usually implicate changes in the cytoskeletal pattern and actin filaments (Rao and Kristen, 1990). In an experiment on *Lens esculenta* L., seed germination decreased noticeably with the applications of Cu, Cd, Hg and Zn, by hampering enzyme activity (Ayaz and Kadioglu, 1996). In two different studies on the effects of Al and Hg on meristematic root cells of *Allium cepa* L. and *A. sativum* L., the results proved that heavy metals inhibit root growth and decrease the mitotic index and cause mitosis anomalies (Kaymak, 1996; Adar and Uysal, 1997). The effects of Cd, Co, Cu, Fe, Hg, Mn, Zn and Al on the ultra-structure and pollen tube growth of *Lilium longiflorum* has also been studied and the highest rate of toxic effect was reported by Cd, Cu and Hg (Sawidis and Reiss, 1995). Compounds with Hg among heavy metals prevent DNA replication and protein synthesis, causing mitotic anomalies and that Cu has similar effects causing chromosomal anomalies (De Flora *et al.*, 1994). According to Sawidis (2008) the application of cadmium mainly affected the tip of the pollen tubes. The first tube irregularity observed was the round swollen tip, which progressively extended to the subapical area forming a distinct subapical collar in contrast to the cylindrical shape of the normal one. The presence of cadmium in solution showed greatest toxicity, because it reduced cell wall plasticity and impairs normal cell elongation in growing pollen tubes causing morphological and structural alterations. The microscopic observations demonstrate that cadmium acts primarily on cell wall development in germinating and growing pollen tubes. Wall anomalies caused by cadmium have also been described for some algae, such as *Chara vulgaris* cells, where local wall thickenings or protuberances consisting of disordered micro fibrils were observed after cadmium treatment (Heumann, 1987). In the brown algae *Cystoseira barbata*, cadmium caused dense deposits in the cell wall (Pellegrini *et al*., 1991).This can be explained by the interaction of metal ions with the anionic contents of secretory vesicles and the fact that pollen tube cell walls contain large quantities of pectins and callose but less cellulose (Herth *et al.,* 1974; Röderer and Reiss, 1988; Sawidis and Reiss, 1995). This special feature of pollen tube cell walls may result in a reaction different from that of other plant cells that possess a normal cellulosic cell wall. Consequently, normal growth is inhibited, the cell diameter increases and walls become thicker. Tuna *et al.,* (2002*)* studied the toxic effect of heavy metals like Ni, Fe, Pb, Co, Cd, Hg, Al, Zn and Cu on pollen germination and tube length of tobacco plant. Heavy metals like Cd^{2+}, Co^{2+} and Pb^{2+} adversely affected pollen germination and tube development (Dutta Mudi and Mondal, 2015;Ghanta and Mondal,2016)

In conclusion, the results proved that all heavy metals have negative effects on pollen characteristics; however, the damage to pollen varies with the doses and plant shows some tolerance against different pollutants. It is also evident that the heavy metals like Co, Pb and Cd inhibit *in vitro* pollen germination and tube growth which ultimately affect the pollen viability as well as sexual reproduction.

Acknowledgements

The financial assistance received from UGC as major research project is thankfully acknowledged. Authors are also thankful to the Department of Botany (DST-FIST & UGC-SAP, DRS), Visva-Bharatri for providing the necessary laboratory facilities.

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How to cite this article:

Megha Dutta Mudi and Subrata Mondal. 2016, Effect of Heavy Metal Toxicity on In Vitro Pollen Germination and Tube Growth of Phyllanthus Reticulatus Poir. *Int J Recent Sci Res.* 7(12), pp. 14571-14575.