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Non invasive electro photon image (EPI) analysis to detect chromium toxicity in cotton (*Gossypium hirsutum* L.) cotyledons

NAYAN M. NIMAVAT[1,2], JAGDISHCHANDRA K. MONPARA[1,2], KIRAN S. CHUDASAMA[1], MANISH L.VEKARIYA[2], VIRENDRA J. PATEL[2], VRINDA S. THAKER[1]*

[1]*Department of Biosciences, Saurashtra University, Rajkot - 360005*

[2]*Vimal Research Society for Agro-Biotech, Aji road, Rajkot, Gujarat, India*

**Email : thakervs@gmail.com*

Abstract : The main objective of the present study was to evaluate the scale and scope of the electrophoton image (EPI) analysis technique in agriculture applications for reliable, rapid, and sensitive outputs. This technique is based on electromagnetic energy in unique patterns generated by the object in the given conditions. We used cotton seedlings grown in different concentrations (0-200 μM) of chromium with MS media (1/2 strength) for two weeks. From each treatment, 10 seedlings were harvested and studied for EPI analysis of the cotyledons. The growth analysis in the terms of fresh and dry weights, water amount of root, hypocotyl, and cotyledons were performed. We observed a decrease in the growth parameters with increasing the concentrations of the chromium in the media. The EPI parameters like total intensity, glow area, average intensity, form coefficient, etc., were measured using its software. This technique is user friendly, rapid, and accurate. It can be used to test the toxicity level of the soils for plant growth without using sophisticated instruments; further, it is fast and non-invasive.

Keywords: Chromium toxicity, cotton plant, electro photonic images (EPI), *Gossypium hirsutum* L., growth analysis, non invasive technique

Due to the increase in industrialization and urbanization, land and water resources are severely polluted (Ugwu and Agunwamba, 2020). One of the key environmental problems related to agricultural soils is heavy metal pollution which ultimately affects the soil plant system, enters into the food chain, and finally, it affects humans and animals (Rebhi *et al.*, 2019). The main cause of this pollution in India is due to inadequate agronomy practices like applications of pesticides and fertilizers, and anthropogenic activities like textile, leather, and electroplating industries; where India stands next to China and European countries (Gao and Xia, 2011). The list of heavy metals that are often found in polluted soils, revealed the abundant source of copper, cadmium, lead, and chromium in combination or even individual forms and decrease plant growth (Wuana and Okieimen, 2011). Particularly, Cr contamination in soil is mainly due to industrialization and its higher mobility, Cr can be transferred from contaminated soils to plants and then to human beings (Li *et al*, 2018). In nature, chromium occurs in two forms; as trivalent Cr (III) and hexavalent Cr (VI) in a permissible limit of 200 mg/kg of soil (Adagunodo *et al.*, 2018). In agriculture farms, contaminated soils have been reported with approximately 350 mg/kg soil.

For the studies on the influence of heavy metal toxicity on plants, many sophisticated techniques are being used to show damage to the plant material at the metabolic level against toxicity responses (Martinez and Gill, 2015; Shahid *etal*, 2017; Li *etal*, 2018,). These types of techniques are often expensive and ineffective in the estimation of subtle responses of the bio object. In contrast, many spectroscopy based sensor techniques like Raman spectroscopy, UV Vis, or magnetic resonance sensors are easy to predict the concentration of a heavy metal ion in plant tissue but they are destructive and expensive. The non invasive methods may help to understand subtle changes in a biological object without compromising its integrity. The electrophoton image (EPI) analysis technique is one of them (Korotkov, 2002). The biophysical principles in the investigation of the EPI technique are based on the ideas of quantum biophysics; the basic science behind the technique is the energy status estimation of the bio object under study at an optimal level and its ability to maintain energy homeostasis in the given situations. These inbuilt characteristics of the object permits to conclusion about the differential metabolic status and physical functionality in a holistic view (Korotkov and Korotkin, 2001).

This instrument originated in Russia based on the Kirlian effect (Kirlian, 1949). But now it is upgraded with a better design, compact model, high resolution camera, and much-revised software (Korotkov and Korotkin, 2001). It is also known as Gas Discharge Visualization (GDV) analysis. It is popular in about 63 countries and it has a Russian Certificate of Conformance as a medical device (Korotkov, 2002). It is mainly designed for medical and human studies but now it is also used for plant studies (Sadikovand Kononenko, 2003).

Cotton (*Gossypium hirsutum* L. family Malvaceae) is an important cash crop in tropical and subtropical countries. It is being grown for two important products *i.e.* fiber and lipoprotein-rich seeds. It is known as white gold and accounts for 25 per cent of textile fiber production worldwide. Thus it has proved to be the backbone of the economy of nearly 75 cotton producing countries. Further, the careful performance of sowing methods and time, fertilizers and irrigation practices, and heavy metal accumulation in farmland soil still could hardly improve the yield.

The present research describes the consequences of Cr toxicity on cotton plants, including morphological changes and standardization of non invasive methods for the detection of chromium heavy metal in the cotton plant.

MATERIALS AND METHODS

Certified seeds of cotton (*Gossypium hirsutum* L. var. Solar 76 BG-2) were purchased from the local market. The seeds were washed and treated with (0.01 %) $HgCl_2$ for 1h. After that seeds were washed thoroughly with running tap water for 30 min. It was washed with sterile distilled water 3 times and kept on wet filter paper in dark for 48h for germination. The uniformly germinated seeds were transferred to Chromium trioxide III treatment. From our preliminary experiments, it was observed that seedlings can tolerate up to 250 µM concentration for 3-4 days. After that seedling starts to decay at a 250 µM concentration. Therefore, in the subsequent experiments, Chromium range 0-200 µM were selected. The seedlings were allowed to grow for 15 days. The seedlings grown in various concentrations of Cr were studied for growth and EPI studies to develop a reliable protocol to estimate toxicity response.

Growth analysis

Seedlings grown in the various concentrations of the Cr showed toxic effects from 20 µM and higher concentrations of the Chromium III treatment. In subsequent concentrations, a gradual decrease was noted. The seedling remained viable till the experiment was performed. Growth in the terms of root and hypocotyl length, cotyledon area, and fresh and dry weights were measured in all studied concentrations. The water amount of each organ under study was calculated by subtracting dry weight data from the fresh weight data, in each concentration. The 0 µM concentration of the chromium served as a control. In each data set, 10 replicates were taken and the experiment was repeated 5 times.

EPI analysis -Instrument basic principle and working

In this apparatus, by a vacuum photogalvanoplastic process a thin metal grid with 10-micron wires is evaporated on the bottom surface of the glass plate (Korotkov and Korotkin, 2001). The sequence of electrical impulses from the highly stabilized generator is applied to this grid generating an electromagnetic field around the subject. Under the influence of this field, the subject produces a burst of electron-ion emission and optical radiation light quanta in the visual and ultraviolet range. These particles and photons initiate electron ion avalanches, giving rise to the sliding gas discharge along the dielectric surface. Spatial distribution of discharge channels is registered via glass plate by the optical system with a CCD camera and digitized in the computer. Thus the technique is called the EPI technique and images after processing are called EPI-grams (Korotkov, 1998). This complete process is carried out in dark to avoid light interference during estimation.

EPI analysis

Cotton cotyledons are impaired in size *i.e.* one is large and another small. Therefore, to get detailed information, we have taken EPI for both the cotyledons separately in 5 replicates. The data collected are analyzed statistically to find out the best correlations with EPI parameters, and to conclude noninvasive, sensitivity, and accuracy. From the EPI analysis machine, the electrogram parameters generated like glow area, entropy, form coefficient, total intensity, glow area of the image, entropy by isoline, and average intensity were measured for defining the studied cotton cotyledons in normal and treated conditions.

Processing for EPI and statistical analysis

Ten cotyledons were used for the EPI procedure from each Cr treatment. EPI parameters were used for further analysis. Each treatment was analyzed for the statistical significance of the assay.

RESULTS AND DISCUSSION

Chromium (Cr) is one of the major environmental pollutants in agro ecosystems. Although Cr at a low concentration (0.1-0.5 mM) may promote plant growth, the higher Cr concentration causes distinct toxicological impacts on plants. Cr not only interferes with the basic physiological, metabolic, and enzymatic activities of plants but also competes with the uptake and translocation of essential mineral nutrients (Shahid *et al.*, 2017). In the present study also, the growth parameters are significantly affected by the Cr treatments.

Data on fresh weight (Fig.1) and dry weight (Fig.2) showed the dose-dependent response in heavy metal-treated cotton seedlings. The maximum fresh weight was recorded at 486 mg in the control while the minimum fresh weight was recorded at 50 mg in 200 µM treated seedling (Fig. 1). In control root fresh weight was decreased with increasing concentration of chromium up to 200 µM. A similar pattern was recorded in hypocotyls and cotyledons; in control hypocotyls, fresh weight was recorded at 390 mg and then decreased with increasing the concentration of chromium in 200 µM hypocotyls it was recorded at 80 mg (Fig. 1). In cotyledon maximum fresh weight was recorded at 353.70 mg in control and minimum fresh weight was recorded at 142.10 mg in 200 µM treatment. There is a clear inhibition observed in root tissue and the general dose-dependent pattern was observed in the fresh weight of root, hypocotyls, and cotyledons (Fig.1).

The maximum dry weight was recorded at 19.4, 14.8 and 28.6 mg in control root, hypocotyls, and cotyledons, and minimum dry weight was recorded at 7.8, 9.6 and 19.1 mg in

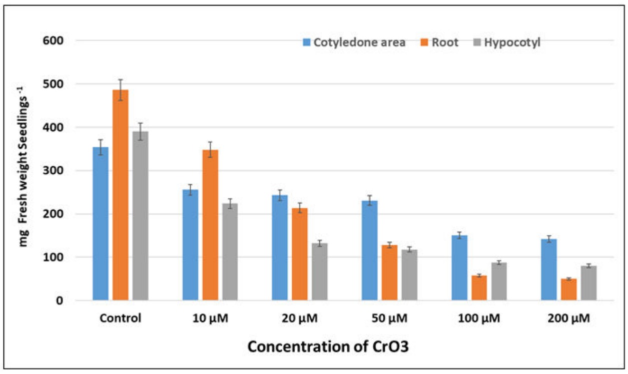

Fig. 1. Changes in fresh weight in the parts of the cotton seedling against CrO_3 toxicity

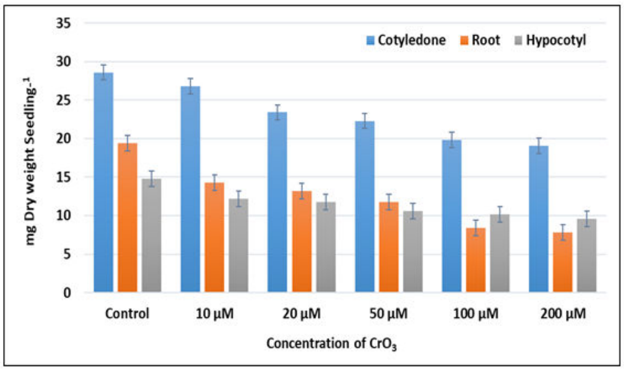

Fig. 2. Changes in dry weight in the parts of the cotton seedling against CrO_3 toxicity

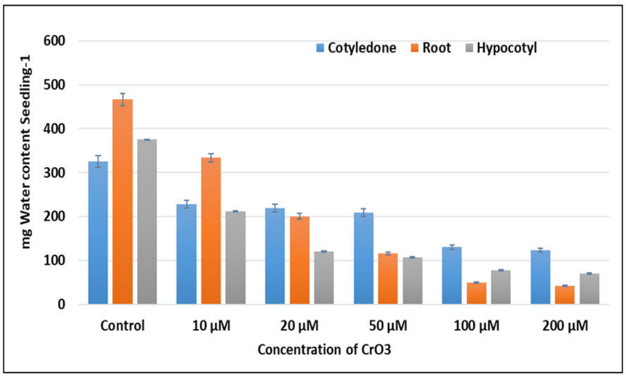

Fig. 3. Changes in water amount in the parts of the cotton seedling against CrO_3 toxicity

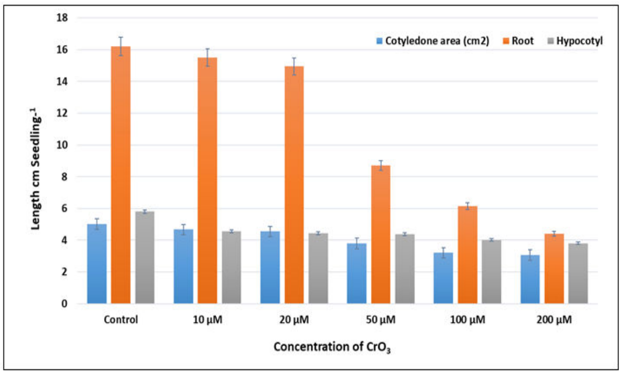

Fig. 4. Changes in the length (root and hypocotyl) and cotyledon area in the cotton seedling against CrO_3 toxicity

200 µM Cr treated seedling organs (root, hypocotyls, and cotyledons), respectively (Fig. 2). Maximum water content was recorded at 466.60 mg (Fig. 3) in the control root, which was the highest among all other treated plants. Water content in root was decreased with increasing the concentration of Cr heavy metal. Water content in 200 µM root was recorded at 42.2 mg. while maximum water content was recorded at 375.2 mg in control hypocotyls and minimum water content of hypocotyls was recorded at 70.4 mg in 200 µM treated seedlings (Fig. 3). Similarly, in cotyledons water content of the control plant was recorded at 325.1 mg per cotyledon which decreased with increasing the concentration of chromium heavy metal in 200 µM cotyledons it was recorded at 123 mg (Fig. 3).

The length of the root and hypocotyls was decreased with increasing concentrations of chromium. In control, maximum root length was observed at 16.2 cm, while in the highest toxic concentration, *i.e.* 200 µM it was 4.41 cm (Fig. 4). It is interesting to note that in each chromium treatment, the lateral root hair development was inhibited significantly. Length of the hypocotyls showed marked inhibition in all concentrations tested. The highest concentration (200µM) showed maximum inhibition. The Cotyledon area was also inhibited by chromium III treatments (Fig.4) cotyledon area in control was 5.8 cm2 and in 200 µM it was 3.81 cm2. Hence the maximum inhibition was observed in 200 µM concentrations. In general, a dose-dependent inhibition pattern was observed in root, hypocotyls, and cotyledons; with increasing the concentration of chromium, the growth decreased but lower concentration *i.e.* 10µM and 20µM was slightly promontory.

It is a common observation in toxicity studies that very low concentrations of heavy metals slightly stimulate growth but higher concentrations are inhibitory. The sensitive concentration or tolerance range varies from plant to plant and even with plant parts. We have also observed marked inhibition in the growth of cotton seedlings parts due to Cr toxicity, but roots are inhibited more than the shoot and cotyledons (Figs.1-4). The roots are first exposed to Cr or any other heavy metals toxicity in the soil. Many reports in the literature showed that Cr toxicity primarily inhibits the growth of the germinating seedlings of Triticumaestivumand Arabidopsis thaliana (Lopez *et al.*, 2014) and it was accumulated in the roots of wheat (Zhang *et al.*, 2010), barley (Ali *et al.*, 2011), rice (Hussain *et al.*, 2018), Viciafaba (Khadra *et al.*, 2019), and Brassica napus (Zaheer *et al.*, 2020) from the polluted soils and damage the crops; ultimately it reduced the yield and also enters in the food chain. Studies on the model plant Arabidopsis seedlings showed that the lower concentration of Cr up to 20 µM-40 µM) stimulates root growth but the higher concentration inhibits it (Lopez *et al.*, 2014). The probable reason for this inhibition may be due to the inhibition in the cell cycle (Sundaramoorthy *et al.*, 2010), reduction of cell division and the primordial formation at Cr 100 µM concentration (Martinez and Gil, 2015) or reduction of root growth under toxicity of Cr may be due to the inhibition of root cell elongation (Mohanty and Patra, 2011) and reductions in tissue uptake of water and required minerals (Liu *et al.*, 2009, Jobby *et al.*, 2018). Similarly, studies reports; inhibition in the shoot growth when Cr is transported from root to shoot, but relatively less inhibition is observed (Nath *et al.*, 2005); however, it damages shoot tissues (Gill *et al.*, 2010). Higher concentrations of more than 50 µM severely influence the growth (Datta *et al.*, 2011). In cotyledons, size, fresh and dry weights, and water amount decreased significantly (Figs: 1-3).

EPI analysis

The data collected are analyzed statistically to find out the best correlations with EPI parameters, and growth parameters for defining toxicity response in the studied cotton cotyledons in normal and treated conditions. It was observed that glow area (GDV area) and total intensity (r2= 0.990; P< 0.001) and, glow area and energy (r2= 0.990; P< 0.001), energy and total intensity (r2=1.000; P< 0.001), energy and average intensity (r2= 0.804; P< 0.001), average intensity and glow area (r2=0.712; P< 0.001), showed positive correlation(Fig. 5). The data revealed that as the Cr concentration increased the glow area total intensity showed an increasing trend. The glow area and length of isoline (r2= - 0.564; P< 0.001) and energy and fresh weight (r2= - 0.694; P< 0.001), fresh weight and average intensity (r2= - 0.762; P< 0.001), fresh weight and total intensity (r2= - 0.694; P< 0.001), showed highly statistically significant negative correlations (Fig. 5). These highly significant correlations can be used to understand the severity of Cr toxicity. The energy values showed a close correlation with the doses of the Cr treatment. Thus Energy value can be taken as a non-invasive marker that is used for the early identification of heavy metal contamination and any other kind of abiotic stresses.

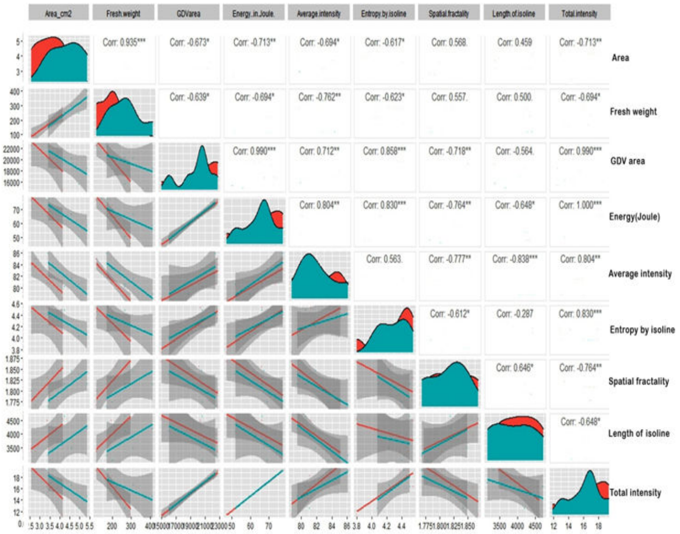

Fig. 5. Correlation between EPI parameters and Growth parameters (*** statistically significant at P≤ .0001, ** statistically significant at P≤ .001, * statistically significant at P≤ .01).

Although, many EPI parameters showed statistically significant correlations with small, large, or both cotyledons (Fig.5); highly significant relations *i.e.* glow area, average intensity, and energy are considered for the scaling of the technique for early and nondestructive detection of trace amount of heavy metal contamination or abiotic stress from the cotton plant. These data of EPI can also be used for the prediction of soil health status and crop yield of plants. Hence with the help of EPI analysis rapid and sensitive detection of the abiotic stress from the cotton plant as well as the contamination status of the growth condition(s) can be detected.

It was demonstrated that the non-invasive EPI technique can provide useful information for some problems in agronomy, *i.e.* distinguishing healthy from stressed or infected plants or differentiating between distinct varieties of the same family of plants (Sadikov and Kononenko, 2003). This technique is also able to point out differences between plants grown using distinct nutrition schemes (Bankovskii, 1986). In addition, the glow area of leaves and fruits revealed useful information about the growth conditions of apples under different treatments (Sadikov and Kononenko, 2003). While the disease-resistant wheat was subjected to an EPI study and the results compared with microfocus radiography showed promising results (Korotkov and Korotkin, 2001). It has unique advantages in terms of both ecological and economic benefits, with great application prospects. The present research will also provide a reference for the comprehensive evolution of the economic feasibility and importance of the EPI technique for the easy and quick detection of tracer amount of Chromium in the early growth stage (seedling) of the cotton crop.

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