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Effects of cigarette tobacco contaminated cultures on germination of maize (*Zea mays* L.) seeds

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Abstract

The maize (*Zea mays* L.) is 2nd most important cereal crop and it is routinely cultivated just before or after the harvest of tobacco crop. Tobacco is the toxic plant mainly used in cigarette, cigars, bidis and chewing in the form of paan and gutkha. The goal of this study is to investigate the toxic effects of cigarette tobacco on seed germination of maize. Seeds are sown on filter-paper wetted with 5% cigarette tobacco in distilled water (T_1), 10% ethanol (T_2), 20% ethanol (T_3) and without tobacco controls like as distilled water (T_0) and 10% ethanol (T_4) for 8-days. A significant effect of cigarette tobacco observed on seed germination. Lowest seed germination, shoot length, root length and plant biomass noted on T_3 culture ($p<0.05$). Similar reduction also measured in chlorophyll contents, total sugars and protein contents, while reducing sugars showed reverse results significantly. Among the cultures T_1, T_2 and T_3 including T_4 (ethanol without cigarette tobacco), the total carotenoids, antioxidants and *proteases* activities noted in increasing trend in comparative to T_0 seedlings ($p<0.05$). It is biochemical signal from the seedlings that their nutrient cultures are growing under nutritional stress. The causes of these cultures are growth inhibitory stressed of heavy metals and other toxic components of tobacco. Cigarette tobacco proved that it is toxic at seed germination stage and could be toxic at both vegetative and reproductive plant growth stages.

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Introduction

In Pakistan, maize (*Zea mays* L.) occupies 2nd position after wheat crop. It has been providing human food, animal's feed, which includes poultry and livestock well. Being a source is extensively used for preparation of various useful energy sources like as cornstarch, corn flakes and dextrose (Khalil *et al*., 2011). Like maize, many other plants are live creature and useful as direct or indirect source of nutrition for living organisms and plants are also competing others for their survival like to animals (Craine and Dybzinski, 2013). Many plants are being harmful for living organisms known as toxic plants (Nagata *et al*., 2010). One of these plants is tobacco (*Nicotiana tabacum* L.), which is consumed from 20-50% of world's population (Kohrman and Benson, 2011; Mac Kay *et al*., 2013).

Main components of complex mixture of tobacco leaf are nicotine, starch, cellulose products, proteins, sugars, alkaloids, hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), phenols, fatty acids, isoprenoids, sterols and many other inorganic minerals. Two groups of them are alkaloid nicotine and isoprenoids (Barceloux, 2012; Hossain and Salehuddin, 2013; Novotny *et al*., 2009; Wahlberg and Enzell, 1987). Many peoples are used to smoke cigarette to burn tobacco, which produces many chemicals. Aired toxic chemicals causes lung cancer, emphysema, chronic bronchitis and heart disease including harmful effect on plants especially agricultural crops (Akinbami *et al.,* 2013; Hecht, 2011; Seimetz *et al*., 2011) and marine and freshwater animals (Zhao *et al*., 2010). Toxic trace and heavy metals are affecting air, water and soil to contaminate environment results into variation in biological responses (Moerman and Potts, 2011; Slaughter *et al*., 2011). Cigarette roadside waste is increasing global environmental load (Booth *et al.,* 2015; Smith and McDaniel, 2011).

Cigarette smoke is also affected and could kill fungus (*Aspergillus* sp. *Mucor* sp. and *Penicillium* sp.) spores (Nautiyal *et al.,* 2007; Zagory *et al*., 1983). It also cause of many diseases in crops (Rath *et al*., 2012). Heavy metals like as arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), zinc (Zn) and other toxic compounds are involved to pollute the soil, which has been up taken by plants (Bleeker *et al*., 2012; Yao *et al*., 2012). It is being dangerous for agricultural environment (Asensio *et al*., 2013; Fowler and Wilby, 2007; Kingwell *et al*., 2006). It is also an alarming point for farmers, who are conscious to rotate crop for better yield. Continuous cultivation of tobacco on the same land could be barren for other crops including tobacco itself.

Tobacco plants including cigarette have toxic effects on seed germination of other plants. All agriculture crops and even seasonal crops cultivated along the tobacco crops also remained affected. This is a rising worldwide problem, could contribute in shrinking of agriculture land with the passage of time. After effects of tobacco includes total plant damage or causes injury in particular parts and tissues. Also changes the physical and chemical soil condition which decreases vegetative as well as reproductive growth of crops plants (Rath *et al*., 2012). In present study, effects of cigarette tobacco on seed germination of maize are investigated. A number of growth parameters observed with limited rates under cigarette tobacco stress. It is dangerous for not only animal's health including human but also toxic for seed germination and its further subsequent growth. This study could be helpful for environment saving strategies by keeping in mind the toxic effects of tobacco plant.

Materials and methods

Plant materials and cigarette

Under subject experiment was conducted in petri-dishes lined with whatman-40 filter paper under sterile control environmental conditions. For this research project, seeds of maize (*Zea mays* L.) variety MMRI-yellow were collected from grain market, District Nawabshah, Sindh. The cigarette pockets (Brand "Dunhill") purchased from local grocer market, Jamshoro. Both were prepared to use them as given below.

Sterilization of maize seeds

Almost 100 seeds of maize were sterilized with 0.1% mercuric chloride ($HgCl_2$) for 10 minutes. Seeds were rinse with sterilize distilled water (dH_2O) for 5-8 times on laminar air-flow cabinet. After washing seeds were collected on sterile filter paper and allow to dry for few minutes. The 4-5 seeds were placed over filter paper per petri-dish, which were already specified with specific treatment or concentration of cigarette's tobacco extract.

Preparation of cigarette's tobacco treatments

The extract was prepared in distilled water, 10% and 30% ethanol solvents. First tobacco leaves were grinded into powder for 3-minutes in grinder. Powder mixture was mixed in above solvents @ 5% (w/v). Mixture was kept at room temperature for overnight. Next day, cigarette tobacco mixture was filtered through filter paper. After filtration, filtrate was preserved at 4°C for next use. Each filtrate was considered as a treatment extract i.e. distilled water extract as T_1, 10% ethanol extract as T_2 and 30% ethanol extract as T_3 against water control (T_0) and 10% ethanol control (T_4). The 2 ml of 4 treatments including water control was poured on filter-paper as lined in petri-dishes separately. All petri-dishes, conical flasks, filter-paper and other stuff were cleaned and sterilized in autoclave at 121°C, 15 lbs/cm² for 15 minutes. After autoclave, sterilized material was dried in electric oven at 65°C for 4-6 hours and opened in laminar air flow cabinet for use.

Seed germination parameters

After fixing the seeds on each treated petri-dishes were incubated at 25°C±2, 25% humidity and 18/6 h day and light photoperiod (light intensity ~2000 lux). Incubation time was 8-days and on each day number of seed germination was counted. On the 8th day, all seedlings were harvested and germination rate was noted. Fresh biomass was taken after measuring the lengths of shoots and roots. Dry biomass was also taken after drying the seedling with electric oven with control temperature at 72°C for 2-days.

Analysis of plant biochemical contents

A number of plant biochemical contents were measure in harvested plant material. For that fresh and dry plant materials were subjected for different analysis as given below.

Chlorophyll and total carotenoid contents analysis

Both chlorophyll and carotenoids were determined in fresh plant material (Arnon, 1949; Lichtenthaler and Wellburn, 1983). Leaves were chapped into small pieces of leaves and mixed in 80% acetone. The mixture was incubated in dark for overnight. On next day, absorbance was read at 663, 645 and 453nm with spectrophotometer for chlorophyll contents, while at 470 nm for carotenoids. Amount of chlorophyll a (*Chl a*), chlorophyll a (*Chl b*) and chlorophyll ab (*Chl ab*) *and carotenoids were* calculated by applying these formulas i.e. *Chl a* = 11.75(A663) - 2.350 (A645), *Chl b* = 18.61(A645) - 3.960 (A663); *Chl ab* = *Chl a* + *Chl a* and *Cc* = [1000(A470) - 2.270(*Chl a*) - 81.4(*Chl b*)] /230.

Determination of total proteins

The total protein contents were measured in fresh plant materials (Lowry *et al*., 1951). Exact 1 ml sample was taken in a test tube than 2.5 ml alkaline copper reagent added. Mixed thoroughly and allowed to stand for 10 minutes at room temperature. The 0.25 ml follin-ciocalteau reagent was added and stand for 30 minutes or till bluish color appeared. Absorbance was measured at 750nm by using spectrophotometer.

Estimation of total sugars

By following the method of Lee and Montgomery (1961), total sugars were measured by taking 0.5 ml sample and mixed in 2.50ml concentrated sulphuric acid (H_2SO_4) and 50µl 80% phenol. Solution mixture was mixed thoroughly and stand for 15 minutes at room temperature and OD was read at 485nm against standard curve of glucose.

Reducing sugars analysis

One milliliter of sample was mixed in 1ml 2, 6-dinitrosalicylic acid (DNS). Mixture solution was heated in boiling water bath for 5 minutes. After heating, sample was cool-down for few minutes at room temperature. Absorbance was taken at 540 nm against standard curve of glucose (Miller, 1959).

Measurements of antioxidant activity (AOA)

The antioxidants activity was determined by following the phosphomolybdenum method (Pisoschi and Negulescu, 2012; Prior *et al.,* 2005; Proestos *et al*., 2013).

The 2.99 ml working solution [9.5 ml 7mM ABTS (2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid), 0.245ml 100mM potassium persulfate, 0.255 ml distilled water (kept at dark for 18 hours in room temperature), diluted with potassium phosphate buffer (0.1M, pH 7.4) until OD reaches to 0.70 (±0.02) at 734nm] was took in clean test tube. Exact 10µl sample mixed thoroughly and reaction mixtures were capped with aluminum foil heated on boiling water bath at 95°C for 90 minutes. Reaction was cooled at room temperature and absorbance was read at 734 nm.

Determination of protease activity

Fresh seedlings material was used to estimate the *protease* activity (Alef and Nannipieri, 1995; Vágnerová and Macura, 1974). The 2.0ml sample mixed with 1.5ml sodium phosphate buffer pH 7.6. The reaction mixture was incubated at 40°C for 25 minutes in water bath. After incubation, 2.0ml 15% TCA (Trichlroacetic acid) was add to stop the reaction. The mixture was centrifuged at 4,000 rpm for 5 minutes. Exact 1ml clear supernatant transferred to new tube than 4.0ml 0.5 NaOH and 1ml follin and ciocalteau phenol reagent (1:1 with water) added. After sometime, blue color was developed than OD was taken at 625nm. For blank reaction, sample was replaced with double distilled water and for standard reaction mixture with known substrate (1% soluble casein).

Statistical analysis of data

The data of this experiment was subjected for analysis of variance (ANOVA) among treatments with CoStat (3.03) *CoHort* software, Berkeley (USA). After data significance analysis, the mean differences among treatments were subjected for further data assessment with Duncan Multiple Range (DMR) test at 5% level (Behrens, 1997; Henley, 1983).

Results and discussion

Maize is the most important cereal food crop and it is cultivated 2-3 times per year to fulfill the need of exponential increasing world's population. With the same time scenario crop rotation system has been encouraged for better yields of crop plants.

Among the monoculture as well as multiple cropping system, allelopathic plants could have harmful or beneficial impacts on next crop. For instance, growth of barley and its seedlings suppressed in rotation to black mustard crop (Tawaha and Turk, 2003). In contrast, continuous cultivation of same crop on the same field gives reduced grain yield (6.2 to 35.4%) substantially (Liu *et al.*, 2012; Pedersen and Lauer, 2004). Tobacco plant also showed allelopathy, as it increases levels of nitrogen, iron, zinc, total soluble phenolics and nicotine in agriculture soil significantly, which caused to decrease in plant growth like as for mungbean crop. The rhizospheric allelochemicals inhibited the biological nitrification inhibition in soil (Norton and Stark, 2010; Subbarao *et al.*, 2006). Among them phenolics root-exuded allelochemicals helpful in solubilization of soil nutrients to release iron and other metallic elements (Badri *et al.*, 2013). Even some allelochemicals decreases nutritional uptake in certain crops affects the growth of plant (Tharayil *et al.*, 2009). The extract of cigarette tobacco decreases seed germination rate as well as its growth rate in maize significantly.

In this experiment, seed germination of maize reduced to 22.22±5.556% in cigarette tobacco extract in 20% ethanol than dH_2O control seedlings (94.44±5.556%). it is also noted lower (72.22±5.556%) in seeds sown in cigarette tobacco extract in dH_2O (Table 1). Similarly, fresh weight of seedling biomass as well as its dry weight also decreased under cigarette tobacco stressed conditions in both of ethanol and dH_2O extracts.

This is further affirmed with the decrease in chlorophyll contents. This confirms differential effects of tobacco allelopathy due to its various constituents especially nicotine. The tobacco plants synthesis nicotine exclusively in root tip (Cai *et al.*, 2013; Dawson, 1942; Li *et al.*, 2007). It is released in rhizospheric region of soil and influences on other plants roots. This study showed reversed results from the plantlets grown in soil (Rizvi *et al.,* 1989) as it is performed on filter-paper lined in petri-dishes. Tobacco extracts inhibited plant growth due to deficiency of chlorophyll contents.

Table 1. Various phenotypes of germinated maize (*Zea mays* L.) seeds after 8-days among the cultures contaminated with cigarette tobacco.

#s	Characteristics	T_0	T_1	T_2	T_3	T_4	*P*-sig.
01	Germination rate (%)	^{a}94.44±5.556	^{b}72.22±5.556	^{c}44.44±5.556	^{d}22.22±5.556	^{bc}61.11±5.556	24.30***
02	Shoot length (cm)	^{a}3.333±0.333	^{ab}2.333±0.333	^{b}1.667±0.333	^{b}1.333±0.333	^{ab}2.333±0.333	5.301*
03	Root length (cm)	^{a}7.667±0.333	^{b}4.667±0.333	^{c}2.667±0.333	^{c}1.667±0.333	^{b}4.333±0.333	41.31***
04	Biomass F.Wt (g)	^{a}1.511±0.045	^{b}0.919±0.002	^{c}0.700±0.017	^{d}0.493±0.038	^{e}0.359±0.029	22.39***
05	Biomass D.Wt (g)	^{a}0.749±0.009	^{b}0.419±0.002	^{c}0.367±0.003	^{d}0.316±0.004	^{b}0.426±0.007	904.2***
06	Chlorophyll a (mg/g)	^{a}3.760±0.038	^{b}2.254±0.049	^{c}1.499±0.026	^{d}0.963±0.072	^{b}2.578±0.025	125.3***
07	Chlorophyll b (mg/g)	^{e}2.098±0.010	^{d}2.250±0.007	^{b}2.499±0.026	^{a}2.601±0.045	^{c}2.304±0.013	58.44***
08	Chlorophyll ab (mg/g)	^{a}5.858±0.031	^{c}4.504±0.055	^{d}3.997±0.052	^{e}3.564±0.110	^{b}4.882±0.014	203.2***
09	Carotenoids (mg/g)	^{d}2.341±0.011	^{c}2.511±0.008	^{b}2.789±0.029	^{a}2.903±0.050	^{c}2.572±0.015	67.43***
10	Total sugars (mg/ml)	^{a}0.672±0.004	^{bc}0.500±0.008	^{c}0.465±0.014	^{c}0.391±0.004	^{ab}0.514±0.008	7.101**
11	R. sugars (mg/ml)	^{c}0.312±0.002	^{b}0.334±0.005	^{b}0.341±0.005	^{a}0.359±0.003	^{b}0.329±0.003	22.07***
12	AOA (mmolTE/g F.Wt)	^{d}0.128±0.003	^{c}0.152±0.002	^{b}0.169±0.002	^{a}0.211±0.004	^{c}0.146±0.003	96.07***
13	*Protease* (Unit/ml/min)	^{a}0.508±0.004	^{b}0.488±0.005	^{c}0.457±0.005	^{d}0.419±0.003	^{b}0.482±0.004	64.98***

T_0: Distilled water control; T_1: Distilled water + 5% tobacco powder; T_2: 10% ethanol + 5% tobacco powder; T_3: 30% ethanol + 5% tobacco powder; T_4: 10% ethanol control; AOA: Antioxidants activity; R. sugars: Reducing sugars; *p-sig*: p significance; F.Wt.: Fresh weight; D.Wt.: Dry weight.

Cigarettes are being used as fashion, habit, most of extent as drug, while its wastes like to tobacco, smoke, liters and butts have toxic effects on plants and other organisms. The acute harmful effects of cigarette butts are increasing day by day for local living organisms (Moerman and Potts, 2011). It is due to changing environmental hazards, which are countinousely increasing (Assunta and Chapman, 2004; Moriwaki *et al.*, 2009). Meanwhile, allelochemicals of tobacco extracts are interfering exactly with plant growth promoters (Mayer and Poljakoff-Mayer, 1963). Variant seedling growth could be the result of level of the extract of cigarette tobacco and the solvent used for its extraction. The inhibition of seed germination is presented in reflection to the in-activation of growth enzyme in the presence of cigarette tobacco in seed germination medium (Matsumoto *et al.*, 2006; Sabharwal *et al.*, 1975; Zhang *et al.*, 2017).

Among the seedlings total carotenoids, antioxidants and protease activity are increased in the medium supplemented with tobacco extract as well as on ethanol (Table 1). In comparison to growth rate of the seedling from normal to tobacco stressed cultures reflects the level of applied stress (Pérez-Alfocea *et al.*, 1993; Raven, 1985). Obviously, it is dependent on the supplied nutritional medium, which are involved in specific alteration on the basis of related interactions (Abhilasha *et al.*, 2008; Grutters *et al.*, 2017; Meiners, 2014; Prati and Bossdorf, 2004). Assembling of new complexes or inhibition or slower down of biological processes are new insights to initiate a specific paradigm shift which helps for the understanding of allelopathy. These un-relevant and non-directional uptake of compounds from the cigarette extract causes significant effect on growth of seedlings. Uptake of various metals including nicotine and various metals can acts to inhibit different enzymes, which could have deleterious effects in disruption of different biological pathways including protein synthesis (Huang and Wang, 2010).

Stressed cultures induces a significant reduction in total protein as well as total sugars than normal control seedlings (Table 1). This decline in protein content might be due to induction of various stress tolerant proteins. The stress proteins mainly comprised on a number of antioxidants (Pinhero *et al.*, 1997; Pisoschi and Pop, 2015; Srivastava *et al.*, 2005). The protein contents decreases under metal stresses in plants, even heavy metals induces oxidative stresses in the form of reactive oxygen species (ROS) with development of hydrogen peroxide, superoxide radical and hydroxyl radical (Asghar *et al.*, 2015; Mohan and Hosetti, 1997; Devi and Prasad, 1998; Song *et al.*, 2006).

The ROS may trigger to slow down various synthesis of biomolecules and different physiological processes (Droge and Droge, 2002; Kaushik and Roychoudhury, 2014; Schieber and Chandel, 2014).

To relax ROS, plants have evolved themselves to cope it with antioxidant systems like as superoxide dismutase (SOD) and other peroxidases (POD), which become active with ROS generation (Valko *et al*., 2006; Vlahogianni *et al*., 2007). In could be in reflection to that extract of cigarette tobacco is involved in developing of oxidative stresses in the form of ROS. Both antioxidants and proteases activities also rises in the stressed seedlings of maize to cope the developed ROS. It may be a good competitive reactions among the ROS and SOD as well as POD to save the seedling from death. Meanwhile, seedling growth remained restricted among the stressed cultures than control ones.

Conclusions

In final, it is concluded that cigarette tobacco have toxic effects on maize seed germination as well as on its further growth. The tobacco's allelopathy has strong but differential toxicity in terms to growth retardation of certain crops when grown in rotation. It develop the growth suppressed conditions for the crops cultivated next to tobacco crop. In case of maize as in present experiment, a number of bio-contents including nicotine of cigarette tobacco extract has induced change in chlorophyll contents and slow-down the activities of various enzymes of biomolecule biosynthesis and cell physiological processes. It results into reduction of seedling growth rate. With this conclusion, it is needful to screen out the crops which can grow in normal form if cultivated over the land just after tobacco harvest.

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