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RESEARCH ARTICLE

Karyotoxicity of fungal metabolites in *Trigonella foenum-graceum* L.

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ABSTRACT

Fungal metabolites are known organic compounds produced by diverse group of fungal organisms in infested host tissues as well as in nutrient growth medium, of them primary metabolites induce growth stimulating response while secondary metabolites appear to create disturbances in normal cell metabolism, growth and karyokinesis in mitotic cell cycle of metabolically active meristematic cells causing cytological abnormalities. The metabolites produced in growth nutrient medium at different growth intervals by *Alternaria alternata* (Fr.) Keissler, a serious causal pathogen of leaf spot of *Trigonella foenum-graceum* L, were isolated, confirmed their chemical nature and evaluated for their cytological effects on metabolically active cells of meristematic zone of roots for cultivars, *Kasuri* and *Kranti.* An increase in per cent seed germination and seedling emergence without any abnormalities over control were recorded with five days old metabolites treated seeds. The seed germination rate and seedling emergence declined while percent abnormalities increased with metabolites of longer duration. The metabolites from five day old culture filtrate served as growth promoter while metabolites of longer duration as growth inhibitor. The meristematic cells of the root from treated seeds were reported to multiply abnormally due to appearance of many abnormalities such as Anaphase Bridge, laggards, diagonal metaphase and mis-orientation of mitotic spindles. The metabolites responded uniformly in both the cultivars with the parameters undertaken. The U.V. absorption spectrum TLC and phytochemicals tests for isolated toxins confirmed phenolic in nature.

KEYWORDS

Karyotoxicity, Fungal metabolite, Trigonella foenum-graceum L, *Alternaria alternata, phytotoxic.*

INTRODUCTION

The fungal organisms of diverse groups are known to secrete or excrete a variety of a multitude of low molecular weight bioactive organic compounds during their growth as secondary metabolites of diverse beneficial and detrimental activities to mankind (Brakhage and Schroeckh, 2011). These metabolites at very low conc. directly toxic to microbes but higher dosages creates disturbances in normal karyokinesis in cell division of metabolically active meristematic cells leads to chromosomal alteration or lethality (Bhajbhuje, 2013). Several species of *Alternaria,* a well-known leaf spot causing plant pathogens, produce more than 70 phytotoxins, of these alterotoxins, alternariol, tenuazonic acid; alternaric acid were reported to play a crucial role in determining host specificity and contributing to disease development (Holensein and Stoessi, 2008). Toxin from secondary metabolites penetrates host tissues, directly act on living host cell protoplasm and damage metabolically active cells to influence the course of disease development (Bhajbhuje, 2013).

Trigonella foenum-graceum, one of the oldest known condiment, legume vegetable as well as medicinal plant of family Fabaceae in the recorded history, is indigenous to an area extending from Iran to northern India, but presently cultivated around the globe as food source for its yellow-to-amber colored seeds (Helambe and Dande, 2012) as the seeds are rich source of protein (26.2%), carbohydrate (44.1%), resign & mucilage (28%), fibers (7.2%), and minerals (3%), carotenes vitamins (thiamine, niacin and riboflavin), alkaloids (trigonelline and choline), and low content of fixed & volatile oil, mucilage, saponin, foetid and nicotinic acid. India is leading producer of fenugreek, accounting for over 40% of the globe's total annual output followed by Iran, China and others including Nepal, Bangladesh, Pakistan, Argentina, Egypt, France, Spain, Turkey, Morocco, North & East Africa, Ukraine, Canada and Greece. Lion's share of India's production, accounting for over 80% of the nation's total output is contributed by Rajasthan (Wikipedia, 2013).

In parts of Asia, the young plants are consumed as vegetable and seeds as a spice or both plants employed as herbal medicine. The content of leaves includes protein (4.4%); carbohydrate (6%); minerals(1.5%); high content of iron (16.9%mgm), calcium(0.47%), phosphorus(0.05%), vitamins such as Vit-C(140 mgm), thiamine, riboflavin, niacin and low fat (Helambe and Dande, 2012). Leaves appears aromatic, cooling and mild laxative hence used as an ingredient in some Indian, Persian, Eritrean, Iranian and Ethiopian cuisine. The leaves contain alkaloids like mono, di- tri-methylamine, choline, trigonelline, neurin, betain and sufficient quantity of iron in organic form. Its regular consumption helps keep the body clean and healthy (Kylenorton, 2011). The seeds are used as reminiscent of curry, in the preparation of pickles, vegetable dishes, daals and spice mixes such as panch phoron and sambar powder. The sprouted seeds and micogreens are used in salad while the roasted seeds reduce bitterness and enhance the flavor (Wikipedia, 2013).

In herbal medicine, *Trigonella foenum-graceum* L is appeared to be effective lactation stimulant and has been proved promote breast milk supply in lactating women (Tiran, 2003), to reduce cholesterol, and enhance stomach function in food absorption and to treat indigestion, delayed labor, common cold, control high blood sugar (Kylenorton, 2011). Both plant parts, leaves and seeds are proved carminative, demulcent, deobstruent, emollient, expectorant, febrifuge, galactogogue, hypoglycaemic, laxative, parasiticide, restorative, uterine tonic, anticholesterolemic, anti-inflammatory, and antitumor (Helambe and Dande, 2012). It is reported to have anti-diabetic, anti-fertility, anticancer, anti-microbial, anti-parasitic, anti-nociceptive properties and hypocholestero-laemic, thyroxine-induced hyperglycemia effects (Wikipedia, 2013). Application of leaves poultice cures external and internal swelling while topical dosages of boiled and butter fried leaves are proved effective in biliousness (Kylenorton, 2011).

The seeds are bitter, mucilaginous, demulcent diuretic and can be a well substitute for cod liver oil. As the seeds are rich source of iron, becomes useful remedy in treating anemia, restores deadened senses of taste; a gargle from seeds is proved beneficial in sore throat. The seeds have soothing effect on the skin and mucous membranes, relieve irritation of the skin, and alleviate swelling and pain. Seed decoction is employed in the treatment of colic, flatulence, dysentery, diarrhea and dyspepsia (Helambe and Dande, 2012). Application of seed powder on head promotes hair growth and prevents their falling off (Kylenorton, 2011). Tea made from seeds acts as quinine in reducing fevers; helps the body to perspire, dispel toxicity and shortens the gestation period of fever. It soothes inflamed stomach and intestines, cleansing the stomach, bowels, kidneys and respiratory tract of excess mucus. It is used as remedy in healing peptic ulcers, as the mild coating of mucilaginous matter deposited by fenugreek provides a protective layer for the ulcers when it passes through the stomach and intestine whereas the leaf tea is used as a douche liquid in treating leucorrhea and in blood formation, and said to prevent puberty complications (Kylenorton, 2011).

An opportunistic pathogen, *Alternaria* species remains as an increasing threat to legume crops around the globe including India, causing leaf spot and other diseases on over 380 plant species (Ambuse, 2013).The susceptible cultivars of *Trigonella foenum-graceum* L are affected with several seed borne diseases, among them *Alternaria* leaf spot, is a serious, incited by *Alternaria alternata* (Fr.) Keissler causing damping off of seedlings, producing brown to black leaf spots lead to a reduction of leaf surface area, adversely affect anabolism, premature defoliation and reported reduction in annual productivity to the extent of 20-30% (Ambuse, 2013). The pathogen has been reported to attack brassica, chili, papaya, wheat, black gram, rice, and sorghum causing leaf spot, rots and blight diseases (Rathod, 2012). The pathogen remain associated with infected seeds with spores on the seed coat or mycelium under the seed coat, grows profusely in favourable environment and causes physiological damage to the seeds during refrigerated transport and storage (Bhajbhuje, 2013). It can also cause upper respiratory tract infection and asthma in people with sensitivity (Wikipedia, 2013). Agalave (1986) studied the karyotoxicity in *Triticum aestivum*; Sung et al (2011); Bhajbhuje (2013) and Tsuge et al (2013) have studied role metabolites by *Alternaria* species in plant system. Presently specific role of fungal metabolites on karyokinesis in mitotic cell division has so far not been reported from *Trigonella foenum-graceum* , it seemed to be worthwhile to study this phenomenon using *Alternaria alternata* (Fr.) Keissler metabolites with meristematic cells of two cultivars of *Trigonella foenum-graceum* L.

MATERIALS AND METHODS

The seed samples of two cultivars of *Trigonella foenum-graceum* L. were obtained from different cultivators and also from local market. *Alternaria alternata* (Fr.) Keissler was isolated from these seeds as internal seed borne pathogen and from infected leaves following the technique of ISTA (2012). Richard's broth and Czapek's broth medium were used for metabolites production. The metabolites were isolated from both culture filtrate and from infected leaves in different duration following method described earlier (Bhajbhuje, 2013).

The same fungus was transferred aseptically into 35ml Czapek's broth medium in 150 ml conical flask and incubated for a period between 5 to 30 days at an interval of five days at 25 ± 1^{0}C. Separate sterilized broth and sterile distilled water were kept as control. Seeds were soaked for 6 hours in sterile distilled water and in different duration of *Alternaria alternata* (Fr.) Keissler metabolites in triplicate. Washing of the seeds was carried out immediately after the metabolic treatment. Hundred seeds were then placed in blotting paper folds for germination and seedling growth studies and other fifty seeds were placed on moist filter paper in sterilized petri-plates for cytological study. The petri plates having seeds were allowed to incubate at 25±1^{0}C in B.O.D. incubator aseptically to avoid spoilage by any saprophyte contaminants. The moisture content of blotter paper containing seeds has been maintained by addition of sterile distilled water when required. The cytological studies were performed following the procedure described earlier (Bhajbhuje et al, 1992). The seedling height was measured and per cent seed germination was recorded on twelfth day. The seedlings raised from germinating seeds were classified into normal and abnormal seedlings whereas ungerminated seeds were categorized into hard and dead seeds. Several parameters such as colour reactions with different reagents, U.V. absorption spectrum and other confirmative phytochemical tests were performed for the toxins isolated from the nutrient broth and infected leaves (Bhajbhuje et al., 1992).

RESULTS AND DISCUSSION

Microbes are ubiquitous and constitute largest group of living creatures with varying potentials in biochemical, physiological and nutritional mode and play a key role in numerous fields including agriculture, biotechnology and biological engineering (Brakhage and Schroeckh, 2011). These microbes either release or excrete many chemically known metabolites during their growth in favourable environment due to constantly occurring diverse metabolic reactions in every functional cell, which at low conc. directly toxic to microbes, may enhance growth of plant seedlings as growth promoter. Its higher dosages may induce stunted growth creating disturbances in normal karyokinesis of cell cycle leads to chromosomal alteration or cause lethality in eukaryotic plant cells (Bhajbhuje, 2013) and also may acts as mutagens results to mutants that exhibit appearance of some phenotypic variations in resultant seedlings of plants in subsequent generation (Nowicki et al., 2012).

The toxins isolated from the nutrient broth and infected leaves have been screened following various phytochemical tests to confirm chemical nature. The colour reaction of the spots to various detection reagents gave a yellow fluorescence under U.V. The colour of the spot on chromatogram was brick red with alkaline diazotized sulphuric acid, blue with Folin-Ciocaten reagent and yellow with alcoholic bromophenol blue. The U.V. absorption spectrum of the compound gave a peak between 250 and 265 nm wavelengths. These phytochemical confirmative tests and the U.V. absorption spectrum revealed that the toxins isolated might be organic compounds of phenolic in nature. Similar type of toxin was isolated from *Alternaria brassicae* (Bhajbhuje et al., 1992); *Alternaria* sp (EFSA, 2011); *Alternaria alternata* (Chung, 2012); *Alternaria brassicicola* and *Alternaria raphani* (Nowicki et al., 2012); and *Alternaria solani* (Bhajbhuje, 2013).

The per cent seed germination, seedling emergence and cytological abnormalities were recorded for each treatment and control for cultivars*, Kasuri and Kranti* of *Trigonella foenum-graceum* L. (Table 1).

In plants, meristematic cells of root and shoot meristem are characterized by comparatively larger nucleus with nutrient rich nucleoplasm, have greater potential of cell multiplication by mitotic division provided favourable climate environment. Many cytological abnormalities were observed in treated seeds such as Anaphase Bridge, fragment, diagonal metaphase and mis-orientation of mitotic spindle (Fig.4). Seed germination rate and seedling height was recorded to enhance by 6.5-7.0% and 8.8-9.6% over the control respectively in both cultivars with five days old metabolites treatment and no abnormalities were observed (Table 1).

Table 1 : Effect of *Alternaria alternata* (Fr.) Keissler metabolites on seed germination and seedling vigour on two varieties of *Trigonella foenum-graceum* L.

Duration of treatment (Days)	Per cent germination[1]		Seedling height[2] (cm		Per cent germination[1]	
	cv. Kasuri	*cv. Kranti*	*cv. Kasuri*	*cv. Kranti*	*cv. Kasuri*	*cv. Kranti*
5	87.67 (+06.49)[3]	92.33 (+06.95)	4.92 (+08.85)	5.13 (+9.62)	-	-
10	75.99 (-07.70)	80.99 (-06.56)	4.14 (-8.41)	4.36 (-6.84)	6.22 ± 0.03	6.76 ± 0.06
15	65.33 (-20.65)	68.33 (-21.16)	3.72 (-17.70)	3.92 (-16.24)	7.57 ± 0.04	7.45 ± 0.03
20	59.33 (-27.94)	62.33 (-28.08)	3.35 (-25.88)	3.45 (-26.28)	9.96 ± 0.05	10.11 ± 0.04
25	51.66 (-37.25)	54.99 (-36.55)	3.11 (-31.12)	3.19 (-31.83)	12.27 ± 0.03	12.68 ± 0.06
30	44.33 (-46.16)	48.66 (-43.86)	2.68 (-40.71)	2.97 (-36.54)	16.88 ± 0.05	17.11 ± 0.05
Czepak's medium	82.99	86.25	5.31	5.41	-	-
Control (D.W.)	82.33	86.67	4.52	4.68	-	-
C.D.(0.05)	1.2	1.6	1.1	1.5		

1. Average of 300 germinated seeds;
2. Average of 100 seedlings;
3. Values in parenthesis indicate per cent reduction or increase in term of control.
4. ± indicates standard error.

Fig. 1: Effect of *Alternaria alternate* metabolites on seed germination in *Trigonella forenum-graceum* L.

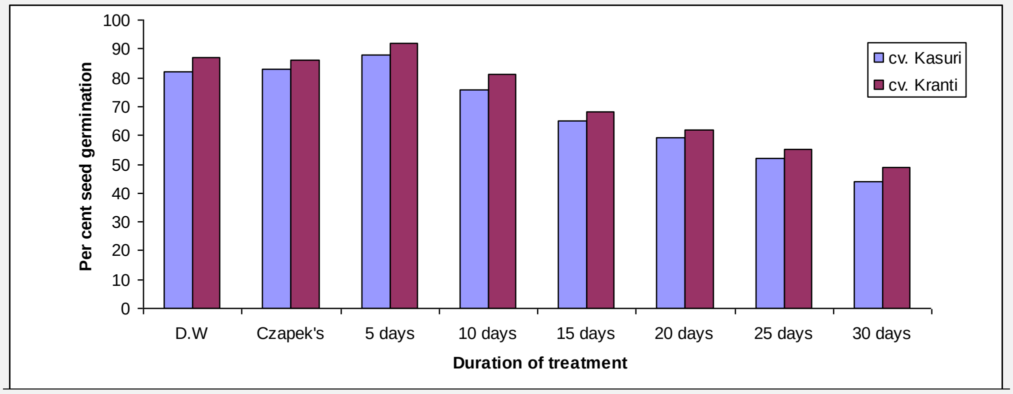


Fig. 2: Effect of *Alternaria alternate* metabolites on seed emergence in *Trigonella forenum-graceum* L.

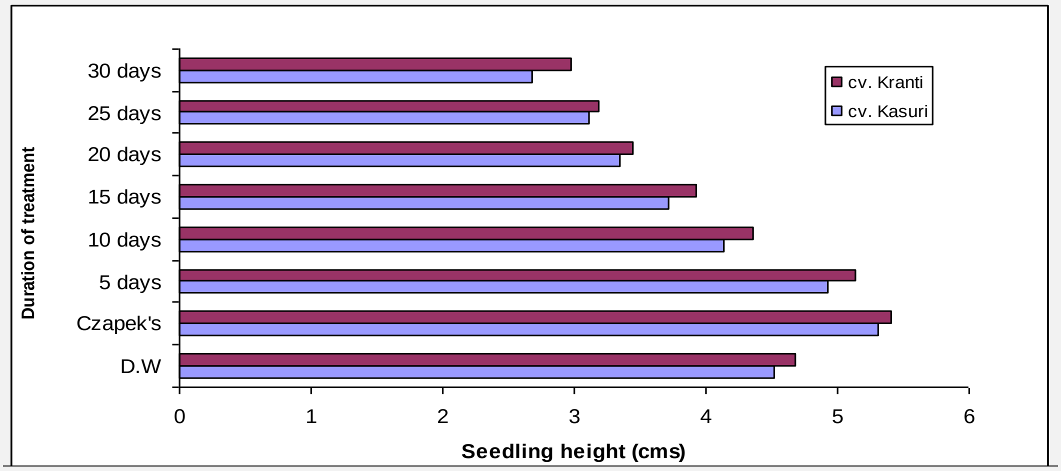
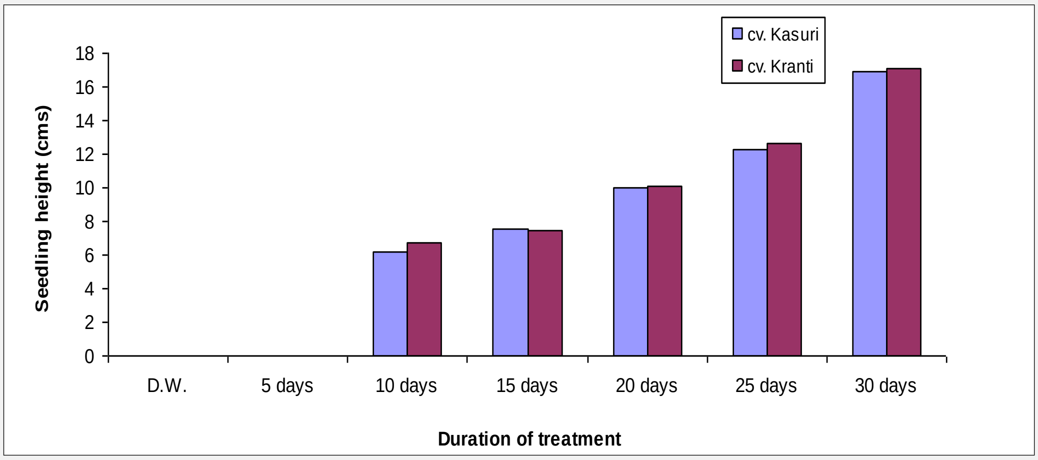


Fig. 3: Effect of *Alternaria alternate* metabolites on seedling emergence in *Trigonella forenum-graceum* L.



a. Mis-orientation of mitotic spindle **b.** Anaphase bridge **c.** Late anaphase showing laggard

Fig. 4: Chromosomal abnormalities in meristematic cell of *Trigonella foenum-graceum* L.

Similar finding to all these parameters were reported in *Triticum aestivum* (Agalave, 1986); *Brassica oleracia & B. campestris* (Bhajbhuje et al., 1992); *Lycopersicon esculentum* Mill (Bhajbhuje, 2013) with five days metabolite treatment. Sung et al., (2011) reported higher seed germination and seedling growth rates in Canola than distilled water and potato-dextrose broth in cucumber and tomato plants receiving treatment of culture filtrate of *Shimizuomyces paradoxus*, a fungal pathogen of mature *Smilex sieboldi* fruits. Moreover, a conidial suspension of $1.0x10^4$/ml resulted in the highest growth stimulating effects on the total plant length, and fresh and dry weight of shoots and roots in cucumber, when compared to highest suspension concentration. Lo and Lin (2002) reported secretion of growth regulating factors by *Trichoderma* sp that increase the rate of seed germination, seedling emergence and increase P, Fe, Zn Mn concentration in cucurbitaceous plants. Chung (2012) reported secretion of primary metabolites by *Alternaria alternata* (Fr.) Keissler at early stages of fungal growth. These primary metabolites at low concentration served as growth promoter, and induced vigorous growth by stimulating phosphorylation in the tissues in the host tissues in the presence of Ca^{2+} and Mg^{2+}(EFSA, 2011). Moreover, the low concentration of these metabolites did not express any phenotypic variation in seedling receiving treatment. A growth stimulating effect in response to seed germination rate and seedling emergence over control in present study may be attributed to secretion of primary metabolites by pathogen at early stages of its growth that may serve growth promoters. Siderophores produced by microbes improve nutrient acquisition, hormonal stimulation, disease suppression and the induction of resistance (Sung et al., 2011).

Per cent seed germination was recorded to decline by 8-46%; and 7-44%; the seedling height was reduced to the extent of 8-41%; and; 7-37% whereas the abnormalities were found to increase by 6-17%; and 7-17% over the control to cultivars *Kasuri and Kranti* respectively when seeds treated with 10 to 30 days old metabolites (Table 1). Control seeds did not express any change. These results were confirmed with the results obtained by Agalave (1986) in *Triticum aestivum* L.; Bhajbhuje et al., (1992) in *Brassica. oleracia & B. campestris* L. and Bhajbhuje (2013) in *Lycopersicon esculentum* Mill. Nowicki (2012) has reported the stunted growth of seedlings receiving treatment with secondary metabolite treatment in crucifer plants. The phenomenon indicates that metabolites are both toxic and mutagenic as far as the present plant material is concerned.

Mycotoxin secretion by several filamentous fungi has been reported in many crops including cereals, vegetables, oil-seed crops and pulses (Holensein and Stoessi, 2008). Host-selective toxins (HSTs) produced by fungal plant pathogens are low-molecular-weight secondary metabolites with a diverse range of structures that function as effectors controlling pathogenicity or virulence in certain plant-pathogen interactions (Tsuge, et al 2013). *Alternaria* species can invade crops at the pre- and post-harvest stage and cause considerable losses due to leaf spot, early blight, rotting of fruits and seeds, may results to secretion of a range of mycotoxins as well as other non-toxic metabolites under favourable environment in cereals, mandarins, peppers, apples, sunflower seeds, oilseeds rape, olives, various fruits and vegetable seeds (Wikipedia, 2013). Amongst other species, *Alternaria alternata* (Fr.) Keissler produced several toxic metabolites of major toxicological importance including, HST-toxin, AAL-toxins, tenuazonic acid, alternariol monomethyl ether, alternariol, altenuene, and altertoxin I (Helambe and Dande, 2012) in artificial nutrient medium during its growth period provided favourable climatic environment. Alternariol and alternariol monomethyl ether also have been produced by pathogen in artificially mould-infested building materials (Chung, 2012). The pathogen had seven pathogenic variants producing different host-specific toxins (HSTs) and cause diseases on different plants (Helambe and Dande, 2012). The HSTs was reported release from germinating conidia of *Alternaria alternata* (Fr.) Keissler prior penetration of host cell (Tsuge et al., 2013).

Mutagenic and carcinogenic effect of mycotoxins has been highlighted by ESFA (2011); Sung et al., (2011); Nowicki et al., (2012); Chung (2012) and Tsuge et al., (2013). **The** mycotoxins are known to cause chromosomal breakage, create disturbances in normal karyokinesis in mitotic cell division, alter regular metabolism & cell membrane permeability and also induced physiological and biochemical changes in host cells, resulting in the rapid increase of electrolyte loss and decline in the membrane potential of metabolically active meristematic cells of the plant system (Sung et al, 2011; Bhajbhuje, 2013). Mycotoxin responds inducing micro-mutation, cause carcinogenic disorders in experimental animals and also pose variety of health hazards in domestic animals and human beings (ESFA, 2011) Most *Alternaria* mycotoxins induced considerable cytotoxic effects, Altertoxin III is reported highly mutagenic while altertoxins I and II induced low mutagenicity (Helambe and Dande, 2012). Nearly ten plant diseases caused by *Alternaria* species in which host-specific toxins (HSTs) reported responsible for fungal pathogenicity were cytologically confirmed by 4',6-diamidino-metaphase chromosomes following the germ tube burst method (Tsuge et al, 2013). **The** toxin is reported to induce cytological and physiological changes in host cells and alter cell membrane permeability reducing the membrane potential (ESFA, 2011). Tenuazonic acid had antitumor, antiviral and antibacterial activity and it initially inhibited the protein synthesis by suppression of the release of newly synthesized proteins from the ribosomes into a supernatant fluid. Alternariol and alternariol monomethyl ether had foetotoxic and teratogenic effects (Brakhage and Schroeckh, 2011). Alternariol-induced cytotoxicity is mediated by activation of the mitochondrial path-way of apoptosis. Higher dosages of tenuazonic acid had inhibitory effect protein synthesis and negatively affected seed germination (Nowicki et al., 2012). The host-selective toxins (HSTs) produced by *Alternaria alternata* (Fr.) Keissler was appeared to be release from germinating conidia prior to penetration inducing susceptibility to host cells by suppressing the defence reactions. Moreover, *Alternaria alternata* HSTs and four cellular components, plasma membrane, mitochondria, chloroplast and metabolically active enzymes have been reported as primary sites of each HST action, leading to elucidation of the molecular mechanisms of HST sensitivity in host plant (Tsuge et al., 2013).

The low concentration of Altertoxin III, caused negligible damage at early stages, its higher concentration in the nutrient medium with longer duration of growth, reported causing more damage to the leaf surface at a later stage (Sung et al., 2011). Per cent seed germination and seedling height were found to be decline in treated seeds with 10-30 days metabolites (Fig. 1 & 2). The toxicity of fungal metabolites was intensified on longer duration of the treatment may be attributed to the more accumulation of secreted metabolites on longer duration, may induced inhibition in seed germination and seedling emergence with chromosomal abnormalities (Sung et al., 2011; Chung, 2012; Bhajbhuje, 2013). The growth of the isolated pathogen results in changes associated with various cellular, metabolic and chemical alterations, including chromosome aberrations and damage to the DNA, impairment of RNA and protein synthesis, enzymes degradation & inactivation, loss of membrane integrity, lowering of ATP, decline in sugar and protein content, inability of ribosomes to dissociate, changes in nutritive quality, starvation of meristematic cells, increase in seed leaches and fatty acid content, reduced respiration and accumulation of toxic substances which lead to spoilage of seeds(Jyoti and Malik, 2013). On the other hand, the prevalence of active fungal spores in seeds suggests an imminent public health danger since their metabolites (mycotoxins) produced in seeds may lead serious and devastating clinical conditions in the consumers (ESFA, 2011); Nowicki et al., (2012); Chung (2012); Tsuge et al., (2013). Sung et al., (2011) and Bhajbhuje (2013) have also reported close relationship between the duration of treatment and per cent chromosomal aberrations in Canola, *Cucumber, Brassica oleracia & B. campestris, Lycopersicon esculentum* respectively.

CONCLUSION

The present study reveals that the metabolites are organic compounds of phenolic nature produced by *Alternaria alternata* (Fr.) Keissler, a leaf spot insisting fungal pathogen of *Trigonella foenum-graceum*. The primary fungal metabolites are secreted at early stage of growth may serve as growth promoter, induced growth stimulating effect enhancing seed germination rate and seedling growth without chromosomal abnormalities. The toxicity of fungal metabolites was intensified on longer duration of treatment by releasing of toxic secondary metabolites, induced chromosomal breakage and creates disturbances in the normal karyokinesis in mitotic cell division in actively growing treated cells of meristematic zone of the roots. The primary metabolites secreted by pathogen may prove beneficial in crop plant as they induce enhancing growth in plants. The toxic metabolites may be used as mutagens in evolving high yielding mutant varieties of economically important crop plants.

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