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Karyotoxicity of Metabolites from *Alternaria alternata* (Fr.) Keisslerin *Spinacia oleracea* L.

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Abstract:

Fungal metabolites are known biconcave organic compounds produced by diverse group of fungal organisms during growth and metabolism. Metabolites secreted in nutrient medium at different growth intervals by *Alternaria alternate* (Fr.) Keissler, a serious causal pathogen of leaf spot of *Spinacia oleracea* L were isolated, confirmed their chemical nature and evaluated for their cytological effects on metabolically active cells of meristematic zone of roots. An increase in per cent seed germination without any abnormalities over control was recorded with seeds receiving five days old metabolites treatment. Seed germination rate declined while percent abnormalities increased with metabolites of longer duration. Meristematic cells from root of treated seeds were reported to multiply abnormally due to appearance of many abnormalities like Anaphase Bridge, laggards, diagonal metaphase and mis-orientation of mitotic spindles. Phytochemicals tests and UV absorption spectrum for isolated toxins confirmed its phenolic nature.

Keywords:
Alternaria alternata, metabolites, seed viability, phytotoxic, *Spinacia oleracia* L.

Introduction:

Diverse group of Majority of microbes release or excrete various active metabolites during their static growth and proliferation in favourable environment in response to constantly occurring diverse metabolic reactions Higher dosages of metabolites of fungal origin induce stunted growth, creating disturbances in normal cell metabolism and cause lethality and also may acts as mutagensresulting to mutants that exhibit appearance of some phenotypic variations in resultant seedlings in subsequent generation (Venda kumari et al., 2012).

A member of family Chenopodiaceae, *Spinacia oleracea* L., is one of the nutritious, vitamin rich edible vegetable crops of ancient Persian origin. It is native to central and southwestern Asia and grown extensively over winter in temperate regions as it is enrich with calcium, iron content and store house of vitamin A, B_2, B_6, B_9, C, E, K and dietary fibres, antioxidants, flavonoids (Marraiki et al., 2012). The spinach, along with other green leafy vegetables, is considered to be an energy enhancer and a rich source of antioxidants. Asides from being used as leafy vegetable, spinach has several medicinal virtues including anticancer property. Spinach prevent eye disorders; protect and strengthen mucous membrane, respiratory tract, urinary tract, intestinal tract and lymphocyte which fights against infection; can improve skin, fights against psoriasis, acne, skin aging and wrinkles. Vitamin K in spinach contributes

in maintaing healthy nervous system. Flavanoids (anti-cancer phytonutrient) slow down the cell division process of human stomach and skin cancer cells(Wikipedia, 2014).

Majority of *Alternaria* species remains as an increasing threat to several crops around the globe causing several diseases in plants (Wagh et al., 2012; BhajbhujeandPathode, 2014). Among these, *Alternaria* leaf spot and blight of *Spinacia oleracea* L.is serious incited by *Alternaria alternata* causing initially small dark black coloured circular spots with concentric rings which becomes irregular at later stage and turned to appear as blight, causing 20-80% mortality. *Alternaria alternata* has been isolated from spinach leaves and stored seeds and has shown to be seed transmitted. The infected seeds are often shriveled, reduced in size with a brown discolouration of the seed surface and loss the seed germination potential. Tsuge et al (2013) and Bhajbhuje (2014) have studied role of metabolites using *Alternaria alternata* with plant system. Presently specific role of fungal metabolites in karyokinesis of mitotic cell cycle has so far not have been reported from spinach.It seemed to be worthwhile to study parameters concerning to seed germination and cytological abnormalities using *Alternaria alternata*(Fr.) Kaisermetabolites with spinach.

Material and methods:

A composite seed sample in storage of spinach (*Spinacia oleracea* L.) from different geographical regions of Nagpur has been screened for different deformities. A leaf spot causing pathogen of spinach, *Alternaria alternata* (Fr.) Keissler was isolated from these seeds as an internal seed borne pathogen and also from infected leaves employing technique of ISTA (2014). The metabolites were isolated from both culture filtrate and from infected leaves for a period between 5 to 30 days at an interval of five days in Czapek's broth medium. The water soaked seeds were treated with isolated metabolites. Bothtreated and untreated seeds were placed on moist filter paper in sterilized petri-plates for germination and cytological study. Seed germination in terms of percentage was recorded and cytological studies were performed. The colour reactions with different reagents, UV absorption spectrum and other confirmative phytochemical tests were performed to the confirmation for chemical nature of toxin (Bhajbhuje 2013).

Results and discussion:

Seed is critical input for substantial agriculture as it is a container of embryos of a new generation and vehicle for the spread of new life (Saskatchewan, 2013). 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 spots to various detection reagents gave a yellow fluorescence under UV. It was detected brick red with

alkaline diazotized sulphuric acid, blue with Folin-Ciocaten reagent and yellow with alcoholic bromophenol blue. The UV absorption spectrum gave a peak between 250 and 265 nm wavelengths. These tests and UV absorption spectrum confirmed phenolic nature of toxin. Similar type of toxin was isolated from culture filtrate of some notable species of *Alternaria* involving *Alternaria alternata* (Chung, 2012); *Alternaria solani* (Bhajbhuje, 2013) and *Alternari atriticina* (Bhajbhuje and Pathode, 2014).

The per cent seed germination and cytological abnormalities for each treatment and control were recorded for *Spinacia oleracea* L. (Table 1). Manycytological abnormalities such as Anaphase Bridge, fragment, diagonal metaphase and mis-orientation of mitotic spindle were observed in treated seeds. The rate of seed germination was recorded to enhance by 10.3% where as a count of dead seeds was confined to reduce by 14.4% over control respectively with five days old metabolites treatment and no abnormalities were observed (Table 1). It is agreement with earlier finding of Sung et al., (2011) whoreported greater seed germination rates over control in Canola, cucumber and tomato plants receiving metabolic treatment of culture filtrate of *Shimizuomyces paradoxus*. Similar results were confined with 5 days old metabolic treatment of *Alternaria solani* in tomato (Raithak and Gachande, 2013) and *Alternaria triticina* in wheat (Bhajbhuje and Pathode, 2014). The secretion of primary metabolites at early stages of growth by *Alternaria alternata* and *A. solani* has been proved by several researchers (Chung, 2012; Raithak and Gachande, 2013; Bhajbhuje, 2014). These primary metabolites at low concentration may serve as growth promoter and induced vigorous growth by stimulating phosphorylation in the host tissues in association of Ca^{2+} and Mg^{2+}(EFSA, 2011). A growth stimulating effect in response to seed germination rate over control may be attributed to secretion of primary metabolites by pathogen at early stages of its growth. Sung et al., (2011) reported siderophores production by microbes improve nutrient acquisition, hormonal stimulation, disease suppression and the induction of resistance.

The results of table 1 revealed that per cent seed germination declined by 5.3% to 36.9% whereas a count of dead seeds was found to increase by17.1% to 75.9% over the control in seeds receiving treatment of 10 to 25days old metabolites. Control seeds did not express any change. These results are confirmed with earlier findings of Venda kumari et al., (2014) in *Brassica carinata*and *B. braun*and Bhajbhuje and Pathode (2014) in wheat. It is proved that *Alternaria alternata* produced nonspecific toxic metabolites in culture filtrate which inhibit seed germination, root length, shoot length and vigour index of seedlings in vegetables and crop plants (Anand et al., 2008). The phenomenon indicates metabolites are both phytotoxic and mutagenic.

The count of hard seeds was reported declined with increase of dead seeds in seeds receiving treatment with metabolites of longer duration (Table 1). These results are in confirmation with earlier finding in cucumber, tomato (Sung et al., 2011) and wheat (Bhajbhuje and Pathode, 2014). The secretion of cell wall, cellulose tannin and other chemicals degrading enzymes by fungal flora may induce softening of seed testa (Jyoti and Malik, 2013). It may be attributed to the softening of seed coat by series of chemical reactions with seed testa followed by diffusion of metabolites of longer duration to embryonic cells leading to gradual increase in rate of dead seeds. *Alternaria alternata* excreted several toxic metabolites of major toxicological importance including, HST-toxin, AAL-toxins, tenuazonic acid, alternariol monomethyl ether, alternariol, altenuene, and altertoxin I in artificial medium during its growth period (Holensein and Stoessi, 2008).

Phytotoxic and mutagenic effect of mycotoxins has been highlighted by Chung (2012) and Venda Kumari et al., (2014). Themycotoxins are known to cause chromosomal breakage, create disturbances in normal karyokinesis in mitotic cell division, alter regular metabolism and cell membrane permeability and also induced physiological and biochemical changes in host cells leading to rapid increase of electrolyte loss and decline in the membrane potential of metabolically active meristematic cells of plant system (Sung et al, 2011; Bhajbhuje, 2013). Alternariol-induced cytotoxicity is mediated by activation of mitochondrial path-way of apoptosis. Higher dosages of tenuazonic acid had inhibitory effect on protein synthesis that lost seed viability (Chung, 2012). Low concentration of Altertoxin III, caused negligible damage at early stages, its higher concentration in the nutrient medium, reported causing more damage to a leaf surface at a later stage (Sung et al., 2011). Per cent seed germination was found to be decline in treated seeds with 10-30 days metabolites (Table 1). The toxicity of fungal metabolites was intensified on longer duration of treatment may be attributed to more accumulation of secreted metabolites on longer duration, may induced inhibition in seed germination(Sung et al., 2011; Bhajbhuje and Pathode, 2014). The growth of an isolated pathogen on seeds results in damage to the DNA, RNA and protein, enzyme degradation and inactivation, loss of membrane integrity, lowering of ATP, decline in sugar and protein content, inability of ribosomes to dissociate, starvation of meristematic cells, increase in seed leaches and fatty acid content, reduced respiration and accumulation of toxic substances which leads to spoilage of seeds (Jyoti and Malik, 2013). Moreover, deposition of active fungal spores on or in seeds suggests an imminent public health danger since their mycotoxins produced in seeds may lead serious and devastating clinical conditions in the consumers (Tsuge et al., , 2013). Sung et al., (2011) and Bhajbhuje and Pathode (2014) have reported close relationship between

duration of treatment and process of inhibition of seed germination and seedling emergence in crop plants.

Table. 1 - Record of per cent seed viability and cytological abnormalities receiving metabolic treatment to *Spinacia oleracia* L. seeds.

Duration of treatment (Days)	Seed viability				Per cent abnormalities
	Per cent seed germination[1]	Ungerminated seeds (%)	Dead seeds(%)	Hard seeds(%)	
5	72.3 (+10.3)[2]	27.8 (-19.4)	23.1 (-14.4)	4.7 (-37.3)	-
10	62.0 (-5.3)	38.0 (+10.1)	30.8 (+17.1)	7.2 (-4.0)	5.21 ± 0.03
15	58.5 (-10.7)	41.5 (+20.3)	35.4 (+31.1)	6.1 (-18.7)	6.71 ± 0.04
20	53.3 (-18.6)	46.7 (+35.4)	38.5 (+42.6)	8.2 (+ 9.3)	8.72 ± 0.05
25	46.5 (-23.0)	53.5 (+55.1)	44.3 (+64.1)	9.2 (+22.7)	11.41 ± 0.03
30	41.3 (-36.9)	58.7 (+70.1)	47.5 (+75.9)	11.2 (+49.3)	11.41 ± 0.03
Czapek's medium	69.3 (+5.8)	30.7 (-11.0)	24.2 (-11.1)	6.5 (-13.3)	14.89 ± 0.05
Control (D.W.)	65.5	34.5	27.0	7.5	11.01 ± 0.02

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

Conclusion:

The results of present investigation revealsthat primary metabolites are secreted by *Alternaria alternate* (Fr.) Keissler, a leaf spot insisting fungal pathogen of *Spinacia oleracea* L., at early stages of growth may serve as growth promoter, and exhibited growth stimulating effect by enhancing seed germination rate without cytological abnormalities. The toxicity of metabolites was intensified on longer duration of treatment attributed to release of secondary metabolites, serves as growth inhibitor, reduced seed germination rate with many cytological abnormalities and greater count of dead seeds. Primary metabolites may be beneficial to crop plants as they enhance seedling growth in plants. The toxic secondary metabolites may be used as mutagens in evolving high yielding mutant varieties of economically important crop plants.

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