



ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; 10(6): 1096-1105
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www.thepharmajournal.com
Received: 17-03-2021
Accepted: 28-05-2021

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Partial purification and bioassay of pathogenic toxin produced by *Alternaria porri* (Ellis) Cif

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Abstract

Onion (*Allium cepa* L.), rightly called as “queen of kitchen” is being cultivated extensively throughout the country under a wide range of climatic conditions. Onion purple blotch is the major disease caused by *Alternaria porri* (Ellis) Cif., causing economic yield loss. The pathogen meant to produce several toxic metabolites both in culture media and in the infected tissues, which were involved in the pathogenesis and symptom expressions. The toxin was extracted from the pure culture of *A. porri* in *in-vitro* The Liquid Chromatography Mass Spectrometry (LCMS) studies on detection of toxin produced from *Alternaria porri*, indicated the presence of tentoxin. The toxin was used for bioassay at various concentrations (50 to 2000 ppm) on seedlings of Arka Kalyan and Bellary Red and the results revealed the significant toxicity on the host tested. Toxin concentration at 2000 ppm inhibited the seed germination of 86.79 and 65.13 per cent in Bellary Red and Arka Kalyan, respectively and shoot and root inhibition of 26.67 and 52.98 per cent in Bellary Red and 28.15 and 42.19 per cent in Arka Kalyan, respectively. Foliar spray of toxin showed maximum Per cent disease index of 50.67 and 42.00 per cent in Bellary Red and Arka Kalyan, respectively at 2000 ppm toxin concentration.

Keywords: Bioassay, toxin, purple blotch, onion

Introduction

The onion crop (*Allium cepa* L.) belongs to the Alliaceae family, is one of the most important spice cum vegetable crop in India and the world and consumed by the human in large quantities and is characterized by its nutritional, medical and economic values (Al- Haboobi, 2020). The percentage of minerals in the edible part contains 0.32 calcium, 0.183 potassium, 0.068, 0.044, 0.015, 0.00005 Sulfur, phosphorus, sodium and magnesium in milligrams respectively (Matloob, 1980) [13].

Onion is an economically important crop having domestic export and potential source of earning in the country and grown in an area of 1285 ha. with an approximate annual production of 23262 tonnes. In 2018-2019 onion production is recorded to be around 23.62 m.t. as against 23.26 m. t. in 2017-18 (The Economic Times Agriculture, 2019) [25].

Onion purple blotch disease is a serious menace in onion-producing countries of the world (Pandotra, 1965) [18]. The disease is most severe in warm and humid conditions and responsible for foliage dropping 62-92% (Suheri and Price, 2001) [24]. Srivastava *et al.*, (1994) [22] surveyed and observed that the severity of purple blotch in *Kharif* crop and recorded 87.8% PDI under high humid conditions. *Alternaria* includes both plant pathogenic and saprophytic species, that may affect the crops in field or can cause harvest and postharvest decay of plant products (Logrieco *et al.* 2003) [10].

Biosson and Renard (1967) [4] reported the symptoms on the leaf as circular to oval water soaked lesions, later these spots progressed to become oblong. As the fungus advanced and ramified within the host tissue a fresh zone of discoloured tissue was formed around the spots, initially spots were white, but later turned pinkish or purple. The changes in colour start from the centre and gradually progressed towards the periphery, where it changed into light purplish. The transition of color was marked by concentric ring clearly visible to the naked eye. The lesion on the stalk of the inflorescence axis caused girdling and in most cases resulted in the destruction of the stalk. In older spots, the spores were produced abundantly on tufts of conidiophores, which appeared as large black patches over the diseased area measuring 2 to 3 x 1 to 3 cm (Ponnappa, 1970) [19]. *Alternaria* fungal species produce more than 70 toxins but only a small number of them have been chemically characterized so far (Zwickel *et al.*, 2016) [27]. The *Alternaria* species have been well-characterized for the production of different host-

specific toxins (HSTs) and non-host specific toxins (nHSTs) which depend upon their physiological and morphological stages (Meena *et al.* 2017) [14]. Some toxins such as alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA) and altertoxins were described to induce harmful effects in animals, including fetotoxic and teratogenic effects (EFSA, 2011) [6]. Culture extracts of *A. alternata* and also AOH and AME were found mutagenic and clastogenic in various *in vitro* systems (Crudo *et al.*, 2019., EFSA, 2011) [5, 6]. Masiello *et al.* (2020) [12] analysed the *Alternaria* toxins, such as alternariol (AOH), alternariol-monomethyl ether (AME) and tenuazonic acid (TA) with values up to 8064, 14,341, and 3683 $\mu\text{g g}^{-1}$, respectively by using a diode array detector (LC-DAD). So it's quite necessary to upgrade the cause for the pathogenesis during host pathogen interaction by means of toxin identification and bio assay method. Therefore present investigation was carried out to know the toxins produced by *Alternaria porri* and their impact on seed germination and pathogenesis through bioassay and discussed here.

Material and Methods

Partial purification of toxins

The method described by Bhaskaran and Kandaswamy (1978) [3] was followed for the extraction of *A. porri* toxin in the laboratory. Three isolates of *A. porri* from Raichur, Ballari and Koppal were subjected to the extraction and partial purification process. The pure culture of *A. porri* isolated from a monoconidial culture from onion leaves of above said locations was cultured on the potato dextrose broth.

For extraction of toxin, the potato dextrose broth was dispensed at the rate of 50 ml per 250 ml conical flasks. After sterilization, one centimeter disc of the fungus from periphery of seven days old culture which was grown on potato dextrose agar was inoculated into the 50 ml PDB in conical flask and incubated at $27 \pm 1^\circ\text{C}$ for 10 days. Mycelial mats were separated from the broth culture by filtration through cheesecloth and finally it was filtered by using Whatman No. 42 filter paper.

Culture filtrates were centrifuged at 12000 rpm for 10 minutes to remove spores. The supernatant was reduced to 1/5th of its original volume by evaporating at 46°C in hot water bath. Gradually two volume of acetone was added to the filtrate with constant stirring till the precipitation was completed and allowed to stand overnight at 4°C. The precipitation was removed by centrifugation at 2000 rpm for 10 minutes. The acetone was removed from supernatant liquid by hot water bath at 40 °C, the remaining solution was adjusted to 1/5th of original volume with water. This solution was extracted three times with two parts of aliquot of water saturated 1-butanol. The water phase was discarded and butanol phase was combined and kept on hot water bath at 40°C till complete dryness. The residues from butanol phase was dissolved in 100 ml water and extracted three more times with 200 ml of aliquots of water saturated butanol. The water phase was again discarded and the butanol phases were combined and dried. The dried residues from the combined butanol phase were dissolved in 200 ml of water. This solution was extracted twice with 400 ml aliquot of diethyl ether and ether phases were discarded. The water phase was taken to dry by hot water bath. Moisture was completely removed and dried product was stored in clean and air-dried bottle. The toxin was obtained as crystalline brown powder. The characterization of toxin was carried out by employing Liquid

Chromatography Mass Spectrometry (LCMS) methods.

Bioassay of partially purified toxin on onion seedlings and seeds

Bioassay of partially purified toxin on onion seedlings were conducted with different concentrations *viz.*, 2000, 1000, 500, 250, 100 and 50 ppm to find out the pathogenicity on onion seedlings of Bellary red and Arka Kalyan varieties. The healthy (25 days old) seedlings of onion were used and sprayed with 10 ml toxin solution. Each treatment was replicated thrice. A control was maintained by spraying distilled water. Effects of toxin on these plants were recorded based on the symptoms development at 10th day after spraying.

Bioassay of partially purified toxin on onion seeds of Bellary red and Arka Kalyan were conducted by soaking the seeds in 10 ml of 50, 100, 250, 500, 1000 and 2000 $\mu\text{g/ml}$ concentrations of toxins separately for one hour. Later the seeds were spread on moistened blotting paper. Each treatment was replicated thrice containing 30 seeds. Equal number of healthy seeds were soaked in the sterilized distilled water and placed on moist blotter paper, which served as control. Observations such as germination percentage, shoot and root length, disease incidence and vigor index was recorded.

Foliar spray of partially purified toxin from *A. porri* at different concentration of purified toxin *viz.*, 2000, 1000, 500, 250, 100 and 50 ppm were tested to find out the pathogenicity on onion seedlings of Bellary red and Arka Kalyan varieties under glasshouse conditions and recorded the disease severity (PDI) of toxin sprayed seedlings.

Results and Discussion

Partial purification and characterization of *Alternaria* toxin

Several toxins are known to be produced by microorganisms which are responsible for expression of disease in plants. These microorganisms produce toxic metabolites in culture media and in the infected tissues, which were involved in the disease development. Toxins play an important role in pathogenesis and symptom expression (Owens. 1969) [17]. During the toxin analysis Tentoxin was recovered from culture filtrates of *A. porri*. Tentoxin is known as a non specific toxin produced by some *Alternaria* species. Its chemical name is Cyclo [N- methyl- L- alanyl- L- leucyl- (α z) - α , B-di dehydro- Nmethyl phenyl alanyl glycyl] and molecular weight of 414 (Da) and chemical formula is $\text{C}_{22}\text{H}_{30}\text{O}_4\text{N}_4$. The toxin mainly influences the initiation and expression of the symptoms in the host tissue (Fig. 1 and 2). The results are in agreement with Owens (1969) [17] and Strobel (1974) [23], who reported several phytotoxins produced by microorganism are responsible for induction of disease in plants. Similarly, Wood *et al.* (1972) [26] reported that many metabolites in culture media and in plant tissues are involved in the disease syndrome. Miao *et al.* (1990) [16] reported X, altertoxin-1, 2 and alternuene (ALT) from culture of *Alternaria alternata*. Similar findings were observed by Robeson and Jalal (1991) [20] who reported Tenuzonc acid from *Alternaria alternata* on *Beta vulgaris*. Amaresh and Nargund (2005) [2] isolated toxins from *A. helianthi*, the causal agent of sunflower leaf blight and Jyothi *et al.* (2014) [7] found secondary metabolite production from different species of *Alternaria*. Meena *et al.* (2017) [14] characterized for the production of different host-specific toxins (HSTs) and non-

host specific toxins (nHSTs). Topi *et al.* (2019) ^[11] reported the presence of alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA) and tentoxin (TTX) by an LC-MS/MS method in maize and wheat. Masiello *et al.* (2020) ^[12] isolated alternariol (AOH), alternariol-monomethyl ether (AME) and tenuazonic acid (TA) with values up to 8064, 14,341, and 3683 $\mu\text{g g}^{-1}$, respectively, by using a diode array detector (LC-DAD).

Bioassay of partially purified toxin on onion varieties

Effect of partially purified toxin at different concentration *viz.*, 50, 100, 250, 500, 1000 and 2000 ppm was tested on onion seedlings of resistant (Arka Kalyan) and susceptible (Bellary red) varieties of 25 days old healthy seedlings. Results revealed that, after 18 to 24 hrs of spraying there was necrosis of leaves near margin at 2000 and 1000 ppm concentrations. However, slight symptoms were observed at 250, 100 and 50 ppm concentrations in Bellary red. Similar symptoms were also noticed in Arka Kalyan, where in slight necrosis at margin after 24 hrs of spraying was observed. After 18 to 24 hrs, there was increase in the withering of leaf at the margin at 2000 and 1000 ppm concentrations. There was less infection noticed at 250, 100 and 50 ppm concentrations in Arka Kalyan (Fig.3)

The per cent inhibition of seed germination over control was higher at a toxin concentration of 2000 ppm (86.79%) followed by 1000 ppm (67.38%), which were significantly superior than other treatments. The least inhibition of seed germination (16.19%) was observed in lower concentration at 50 ppm. The effect of toxin on resistant variety was also tested. The results revealed that the per cent inhibition of seed germination over control was maximum at a toxin concentration of 2000 ppm (65.13%), followed by 1000 ppm concentrations (56.17%) which were significantly superior over other treatments. The least inhibition of seed germination (7.86%) was observed in 50 ppm concentration. Per cent inhibition of seed germination in both the varieties were positively correlated with the toxin concentration (Fig.4) (Table. 1).

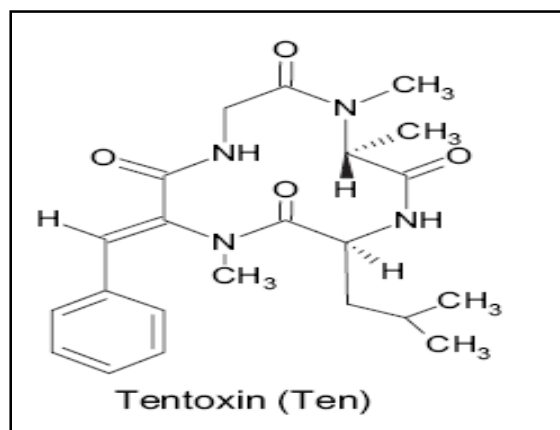
The different concentrations of the toxin on Bellary red variety has reduced the shoot and root lengths significantly. Maximum inhibition of onion shoot (26.67%) and root length (52.98%) was observed at 2000 ppm concentration followed by 1000 ppm concentration (23.00%) and (51.35%) respectively. The least inhibition of shoot (4.30%) and root length (16.29%) was observed in 50 ppm followed by 100 ppm concentrations which showed 7.72 per cent and 16.80 per cent inhibition of shoot and root length over control, respectively. The reaction of Arka Kalyan resistant variety to the toxin was also reported. The results showed that, maximum inhibition of shoot (28.15%) and root length (42.19%) was observed at 2000 ppm concentration followed by 1000 ppm concentration (24.53%) and (33.91%), respectively. Least inhibition of shoot (4.04%) and root length (6.46%) was observed in 50 ppm concentration followed by

100 ppm, which showed 7.36 per cent and 11.58 per cent inhibition of shoot and root length over control, respectively. Influence of toxin on root length and shoot length in both resistant and susceptible varieties clearly showed that, negative correlation with toxin concentration, but per cent inhibition of root length and shoot length positively correlated with toxin concentration (Fig.5a) (Table.1). Toxin effect on seedling vigour was tested and the vigour index was slightly differed between Bellary red and Arka Kalyan. In case of Bellary red, the minimum seedling vigor index was found at 2000 ppm (342.23) followed by 1000 ppm (741.11) and 500 ppm (1107.08) and maximum vigour index was found at 50 ppm (2396.11) toxin concentration. Similarly, in case of Arka Kalyan minimum seedling vigor index was found at 2000 ppm (837.11) and maximum vigour index was found at 50 ppm (3063.67). The different concentrations of toxin inhibited the vigour index of onion seedlings significantly. The vigour index was negatively correlated with toxin concentration. Comparatively more vigour index was noticed in resistant variety compared to susceptible variety (Fig.5b) (Table.1).

Efficacy of toxin was also tested on disease development by foliar spray. It reveals that, maximum PDI was noticed at 2000 ppm concentration in resistant (42.00) and susceptible (50.67%) varieties, but least PDI of 6.67 per cent in resistant variety and 13.33 per cent in susceptible variety at 50 ppm concentration. The toxin concentration has positive correlation with disease development. As per the previous literature Arka Kalyan has claimed as resistant to onion purple blotch disease. In our field study during screening the severity in this variety was quite high and comes under moderately susceptible category. Therefore in bioassay study after toxin spray the Arka Kalyan variety has showed quite higher severity (Fig. 6) (Table.2). The results are similar to the finding of Miaero and Bean (1991) ^[15] who found marginal necrosis, wilting and chlorosis after the treatment with alternaric acid and zinniol. Similar observation was also reported by Khare and Goswami (1996) ^[9], who assayed *A. porri* filtrates on root length and chlorosis of onion leaves. Amaresh and Nargund (2005) ^[2] reported that, toxins from *A. helianthi*, which is related to furon molecule and is 3-propyl, 4-hydroxy 2-oxo-2, 3-hydro furone inhibited seed germination, root and shoot length at 50 ppm and above concentration. The present findings are also in conformity with the results of Savitha *et al.* (2012) ^[21], they found maximum inhibition of seed germination and shoot and root length at 2000 ppm concentration in *Alternaria sesami*. Least inhibition of root and shoot length was found at 50 ppm concentration. Kalamesh *et al.* (2013) ^[8] reported the glycoprotein toxin from *Alternaria helianthi* causing *Alternaria* blight in sunflower shown maximum inhibition of seed germination, shoot and root length at 3000 ppm and least inhibition at 100 ppm concentration. The results indicated that, the seed germination and seedling growth of Bellary red was significantly reduced after treating the seeds with different concentration of partially purified toxin.



Fig 1A): Partial purification of toxin



B) Chemical structure of tentoxin produced by *Alternaria porri*

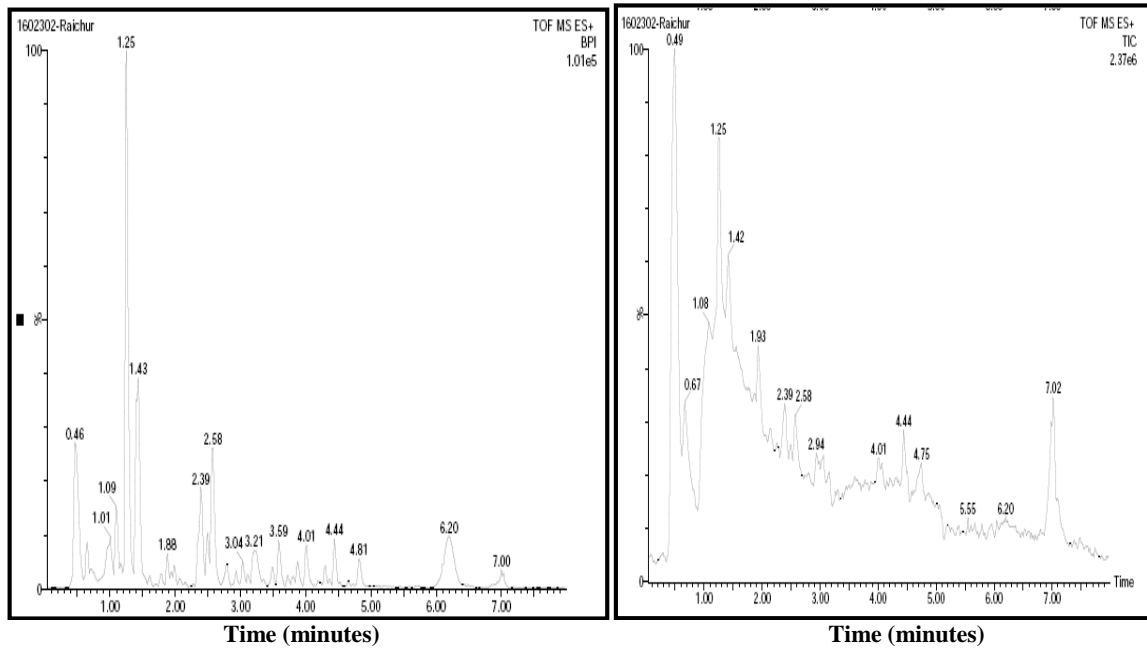


Fig 2: LC-MS detection of purified toxin produced by *Alternaria porri*



Fig 3: Bioassay of partially purified toxin inoculated on Bellary red and Arka Kalyan at different concentrations (ppm) by pin prick method

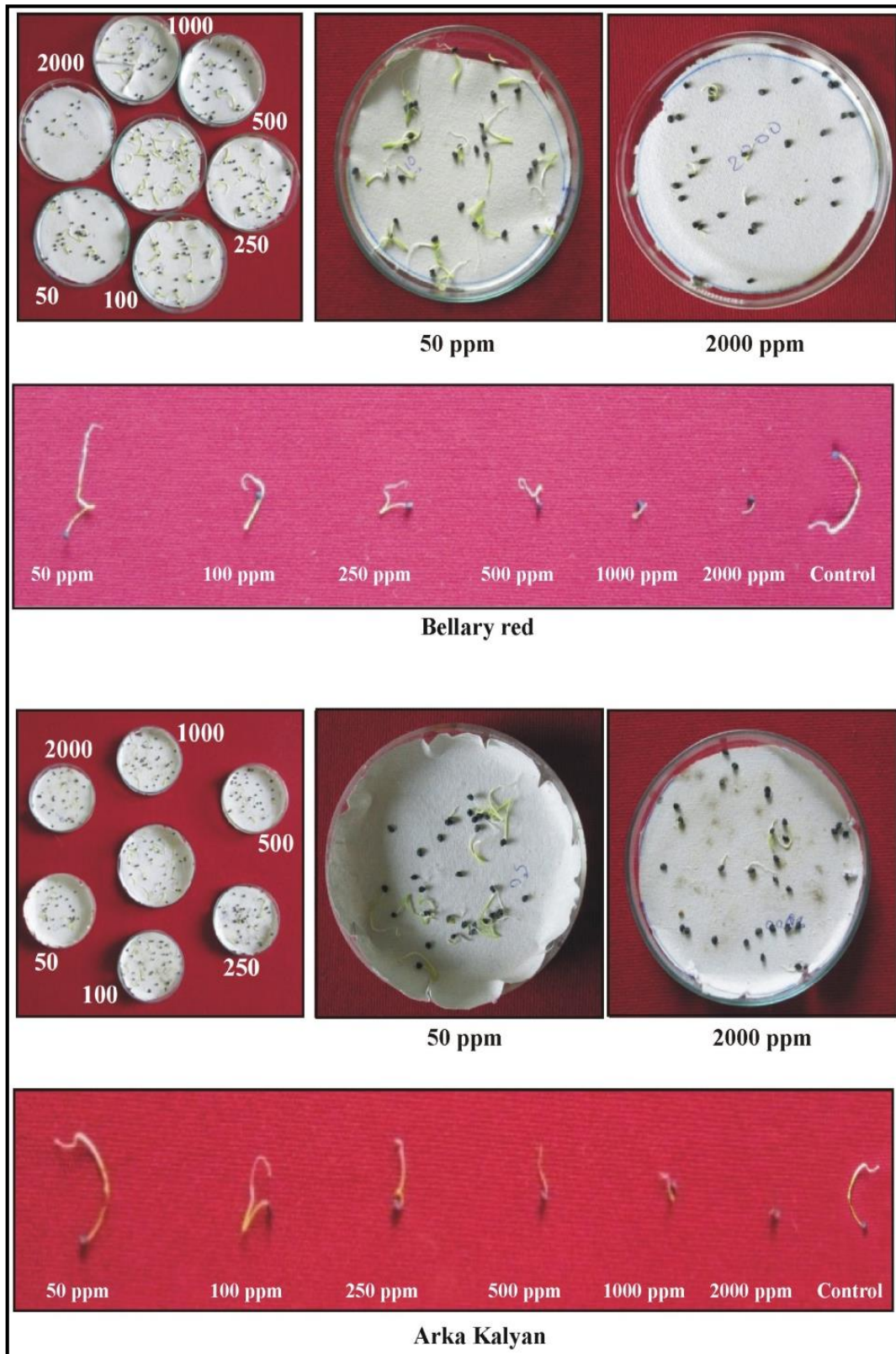
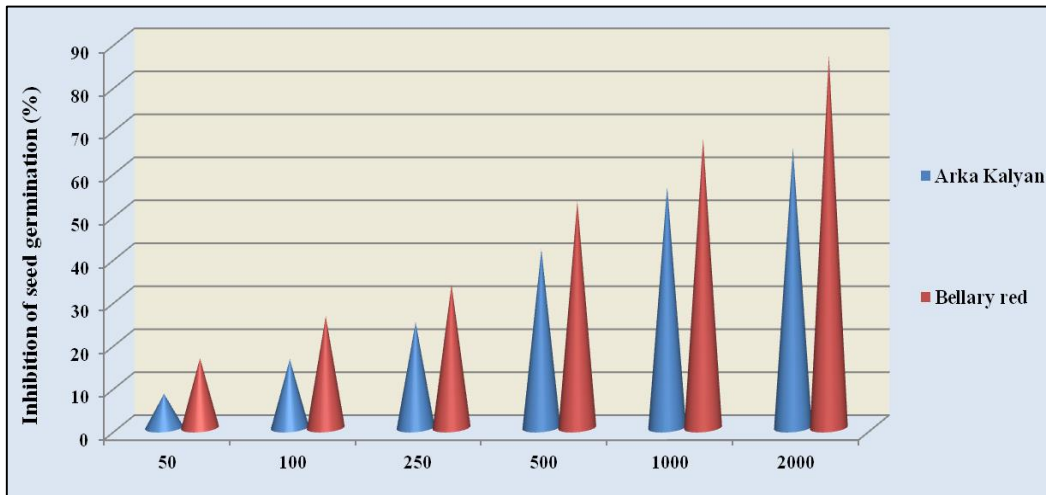


Fig 4: A) Effect of partially purified toxin on seed germination in Bellary red and Arka Kalyan at different concentrations (ppm)



B) Effect of partially purified toxin on inhibition of seed germination of Arka Kalyan and Bellary red varieties

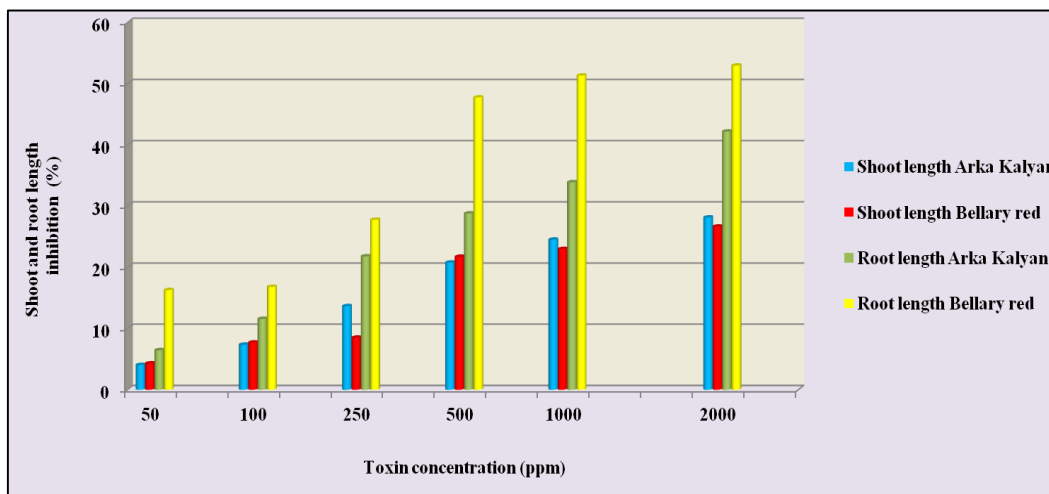
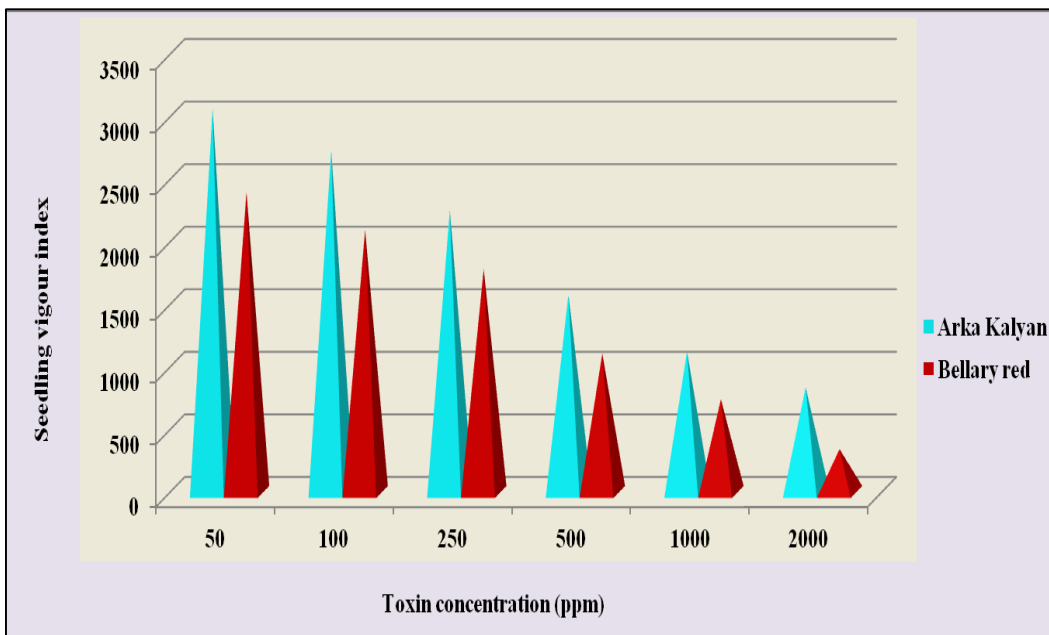


Fig 5A): Effect of partially purified toxin on inhibition of shoot and root length of Arka Kalyan and Bellary red varieties



B) Effect of partially purified toxin on seedling vigour index of Arka Kalyan and Bellary red varieties

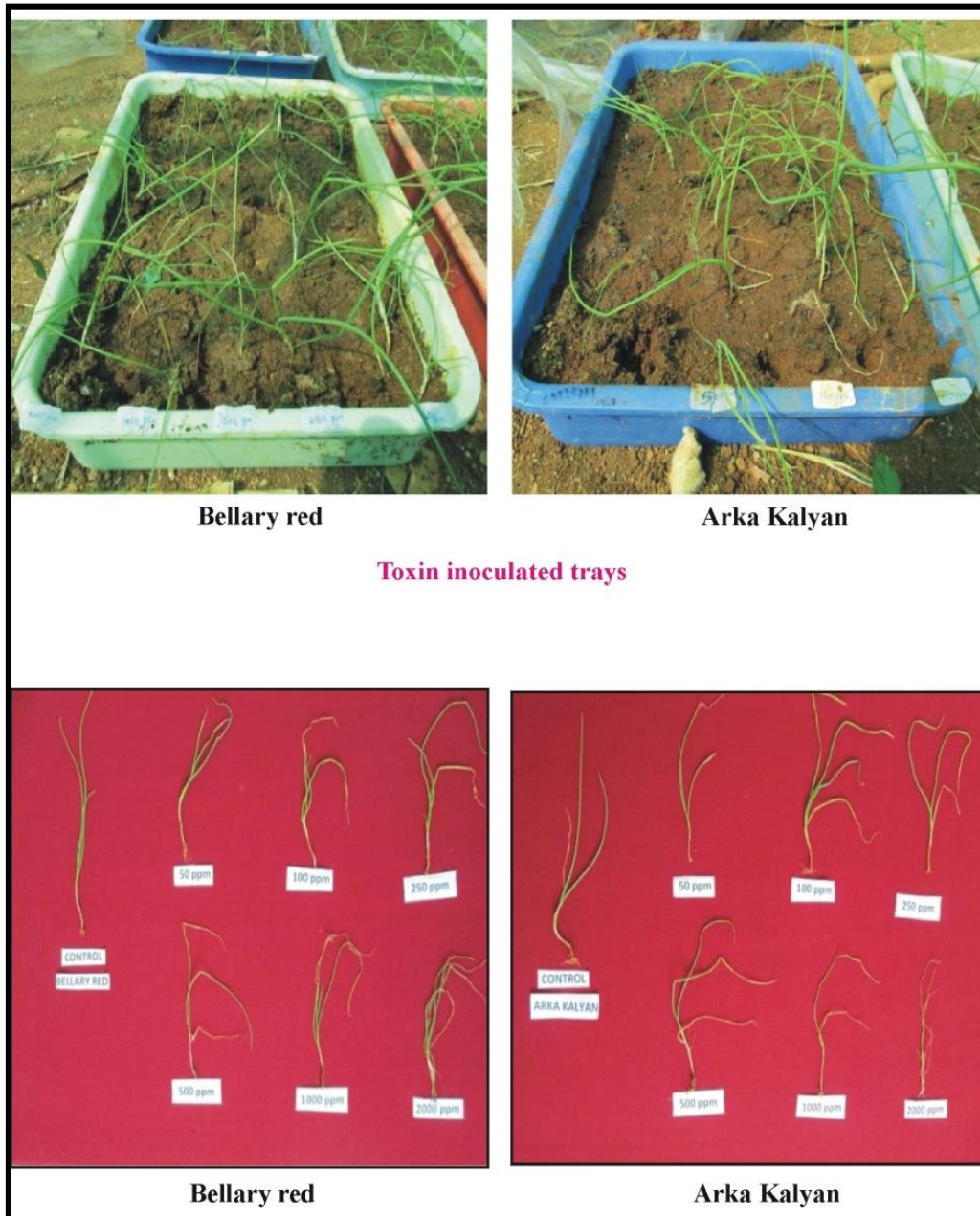
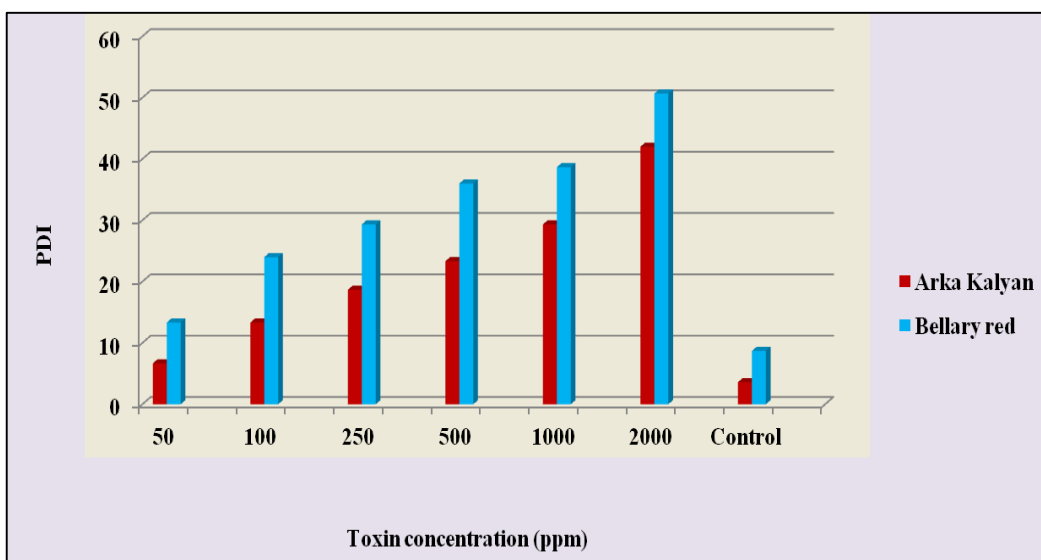


Fig 6A): Bioassay of partially purified toxin on symptom development in Bellary red and Arka Kalyan inoculated by foliar spray



B) Effect of partially purified toxin on PDI of purple blotch of onion

Table 1: Effect of partially purified toxin on seed germination and seedling growth of susceptible (Bellary red) and resistant (Arka Kalyan) onion varieties

Treatment No.	Toxin concentration (ppm)	Inhibition of seed germination over control (%)		Inhibition of seedling growth over control (%)				Mean shoot length (cm)		Mean root length (cm)		Vigour index (%)	
				Shoot length		Root length							
		R	S	R	S	R	S	R	S	R	S	R	S
1	50	7.86 (16.28)	16.19 (23.72)	4.04 (11.55)	4.30 (11.97)	6.46 (14.7)	16.29 (23.79)	26.93	24.00	6.70	5.20	3063.67	2396.11
2	100	15.90 (23.49)	25.94 (30.62)	7.36 (15.74)	7.72 (16.12)	11.58 (19.89)	16.80 (24.19)	26.00	23.07	6.33	5.13	2730.11	2095.33
3	250	24.56 (29.70)	33.38 (35.29)	13.66 (21.69)	8.52 (16.97)	21.79 (27.83)	27.77 (31.80)	24.23	22.87	5.60	4.43	2253.56	1782.67
4	500	41.53 (40.12)	52.76 (46.58)	20.78 (27.12)	21.73 (27.78)	28.8 (32.46)	47.77 (43.72)	22.33	20.46	5.10	3.20	1578.89	1107.08
5	1000	56.17 (48.54)	67.38 (55.17)	24.53 (29.68)	23.00 (28.65)	33.91 (35.61)	51.35 (45.77)	21.18	19.23	4.73	3.00	1122.06	741.11
6	2000	65.13 (53.82)	86.79 (68.69)	28.15 (32.04)	26.67 (31.09)	42.19 (40.5)	52.98 (46.71)	20.17	18.27	4.13	2.90	837.11	342.23
7	Control	---	---	---	---	---	---	28.07	0.39	7.17	0.10	3484.11	2839.20
S. Em ±		0.55	0.44	0.42	0.39	0.26	0.38	0.16	0.39	0.10	0.10	18.27	9.77
C. D. at 1%		2.40	1.90	1.84	1.71	1.16	1.67	0.68	1.65	0.45	0.45	76.91	41.16

* R- resistant variety * S-susceptible variety * Figures in parenthesis are arc sign transformed value

Table 2: Effect of foliar spray of partially purified toxin on severity of purple blotch on susceptible and resistant varieties

Sl. No.	Toxin concentration (ppm)	PDI (%)	
		Resistant	Susceptible
1	50	6.67 (14.93)	13.33 (21.41)
2	100	13.33 (21.40)	24.00 (29.33)
3	250	18.67 (25.59)	29.33 (32.79)
4	500	23.33 (28.88)	36.00 (36.87)
5	1000	29.33 (32.79)	38.67 (38.45)
6	2000	42.00 (40.39)	50.67 (45.38)
7	Control	3.60 (10.70)	8.70 (14.62)
S.Em ±		0.65	0.42
C. D. at 1%		2.83	1.82

* Figures in parenthesis are arc sign transformed values

Conclusion

The above results clearly defines that the isolated toxin compound was lethal to the plant species and involved in expression of symptoms. The bioassay of isolated toxin at various concentrations may cause higher yield loss by means of inhibiting seed germination and seedlings root and shoot growth.

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