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# Isolation of crude toxin, thin layer chromatography (TLC) and HPLC analysis of *Bipolaris oryzae*, inciting brown spot disease of rice

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## Abstract

Brown spot disease (*Bipolaris oryzae*) has been associated with two major epidemics in India and one of the strongest yield reducers among rice diseases in recent years. The disease especially occurs in environment where water supply is scarce combined with nutritional imbalance particularly lack of nitrogen and hence often referred to as 'poor man's disease'. In the present study, six isolates of *B. oryzae* were used for extraction of toxin by solvent extraction procedure. Toxin profile of six isolates of *B. oryzae* on Thin-layer chromatography (TLC) plate showed a total of eight bands; five bands at Rf 0.93, 0.85, 0.77, 0.61 and 0.49 were distinct and all isolates produced a common dark band at Rf 0.61 in iodine chamber at 254 nm. The quantitative analysis of toxin produced by different isolates was carried out by High-performance liquid chromatography (HPLC) analysis. Six isolates of *B. oryzae* showed variation in their peak values at different retention time. In all isolates unique peak with retention time at 4.3 min was observed. This compound which was observed in all the isolates can further be characterized for its property of toxin by using distinct standards.

**Keywords:** Brown spot disease, *Bipolaris oryzae*, Thin-layer chromatography (TLC), High-performance liquid chromatography (HPLC)

Rice is one of the most important cereal crops and feeds more than one third of the world's population (Burgos *et al.,* 2013). Rice is susceptible to several leaf spot diseases like blast and brown spot, which cause significant yield losses across the globe. The yield loss was upto 90% in epiphytotic form at leaf spot phase during the year 1942-43 of Great Bengal Famine (Ghose *et al.,* 1960). When the disease was reported in 1919, extensive research has been carried due to which the disease was controlled. In recent years, because of the climate change and cultivation practices, disease was found to be severe in dry/ direct seeded rice in the states of Bihar, Chhattisgarh, Madhya Pradesh, Odisha, Assam, Jharkhand and West Bengal. It especially occurs in the environment where water is a scarce resource combined with nutritional imbalance particularly lack of nitrogen and hence often referred to as "Poor man's disease'' (Baranwal *et al.,* 2013).

The pathogen attacks the crop from seedling to milky stage. The symptoms appear as minute spots on the coleoptile, leaf blade, leaf sheath and glume, being most prominent on leaf blades and glumes. On leaves, typical spots are brown in colour with grey or whitish centre resembling sesame seed with typical yellow halo over the spot (Sunder *et al.,* 2005). Conidia are 5-10 septate with the oldest conidium towards base. Typically conidia are slightly curved and widest at the middle. The optimum temperature for growth and conidial germination has been found to be 27-30 °C and 25-30°C, respectively (Ou, 1985) wherein conidia are formed between 5-38 °C, optimum being 25 °C (Ou, 1985; Vinay Kumari *et al.,* 1997). Both light and dark periods were required for sporulation of *B. oryzae*. However, it was stimulated by near-ultra violet light and inhibited by blue light (Ou, 1985).

The pathogen is reported to produce phytotoxins in culture. Goto (1958) reported the toxic effect of the culture filtrate of *B. oryzae* on rice plants. Terashima *et al.* (1962) isolated ergosterol from the mycelium of the fungus. Cochliobolin extracted and purified from filtrate and ophiobolin detected

in diseased leaves inhibited the growth of roots, coleoptiles and leaves (Nakamura and Oku, 1960; Ou, 1985). Narain and Simhachalam (1976) claimed that the toxin was completely inactivated by copper oxychloride. Beside these, the pathogen has also been observed to produce host specific toxin, which elicited the characteristic brown spot symptoms (Vidhyasekaran *et al.,* 1986) through suppression of defense mechanism by decreasing phenolic content and phenyalanine-ammonia lyase activity in rice leaves (Vidhyasekaran *et al.,* 1992). Hegazy *et al.* (1992) reported that partially purified toxin (s) from the pathogen inhibited seed germination and reduced root and shoot length in rice cultivars. Ophiobolin A from *Bipolaris oryzae* perturbs motility and membrane integrities of porcine sperm and induces cell death on mammalian somatic cell lines (Bencsik *et al*., 2014).

Morpho-pathological and molecular characterization of *B. oryzae* has been carried out for fifty isolates in India (Kumar *et al.,* 2011). Diversity and pathogenicity of the rice brown spot pathogen were investigated earlier by many workers using morphological characteristics as well as genetic fingerprint analysis in India as well as in other rice growing countries (Motlagh and Kaviani, 2008; Kamal and Mia, 2009; Motlagh and Anvari, 2010; Burgoss *et al.,* 2013, Archana *et al*., 2014, Kandan *et al.,* 2014 and Nazari *et al.*, 2015). Morphological, molecular characterization and grouping of 27 isolates of *Bipolaris oryzae* from India were carried out by Singh *et al.,* (2016). Morpho-molecular diversity for 116 isolates of *Bipolaris oryzae* from different rice growing areas of India was studied by Kumar *et al.,* (2016). Morphological, molecular characterization and grouping of 17 isolates of *Bipolaris oryzae* from India were carried out by Valarmathi and Ladhalakshmi (2018). This paper mainly dealt with the isolation of crude toxin followed by TLC and HPLC studies.

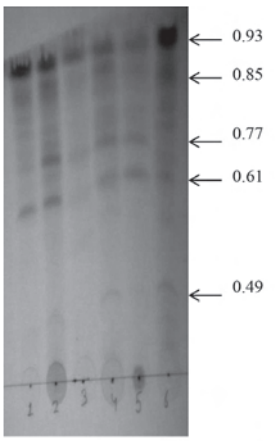
The toxin was extracted from the six *B. oryzae* isolates using solvent extraction procedure. Each isolate inoculated into three replicated flasks (1 L) containing 200 ml PDA medium were incubated at 25 ± 1° for 3 weeks. The culture filtrate was extracted with chloroform taken in a separating funnel. The collected chloroform layer was evaporated by rotary evaporator (55 ºC and 120 rpm) and the residue (crude toxin) dissolved in 1ml of methanol was stored at 4 ºC. 10 µl of partially purified toxin isolated from all isolates was loaded on Silica-gel TLC plates and developed in Benzene: Acetone (1:1) solvent system. Air dried plates were visualized by iodine vapours in iodine chamber and under UV light (254 and 365 nm). Rf values of various compounds resolved on TLC plate were measured (Jahani *et al.,* 2013). The quantitative analysis of toxin produced by different isolates was carried out by High-performance liquid chromatography (HPLC) as per the protocol given by Jahani *et al.,* (2013).

Toxin profile of six isolates of *B. oryzae* on TLC plate showed a total of eight; bands of which, five bands at Rf 0.93, 0.85, 0.77, 0.61 and 0.49 were distinct (Fig. 1). All the isolates produced a common dark band at Rf 0.61 in iodine chamber at 254 nm. HPLC analysis of six isolates of *B. oryzae* showed variation in their peak values at different retention time (Fig. 2a-2f). In the isolate BO 1, four peaks were observed at 4.3, 5.2, 6.1 and 12.2 min, in BO 2 three peaks at 4.2, 5.5 and 12.2 min and in BO 3 three peaks at 4.2, 5.4 and 13.1 min were observed respectively. In the isolate BO 1319 and BO 5326 two peaks at 4.3 and 12.1 min were observed. In the isolate BO 23, various peaks were observed at 4.3, 5.2, 5.5, 6.6, 11.1 and 12.8 min respectively. In all isolates unique peak with retention time at 4.3 min was observed (Table 1). The compound which was observed in all the isolates can further be characterized for its property of toxin.

Crude toxin extracted from the isolates of BO 1, 2, 3, 1319, 5326 and BO 23 were used for thin layer chromatography (TLC). In the TLC, five bands at Rf 0.93, 0.85, 0.77, 0.61 and 0.49 were distinct. Similar studies in *B.sorokiniana* by Jahani *et al.,* (2013) showed Rf values 0.90, 0.81, 0.76, 0.67 and 0.44 distinct wherein 0.44 produced common dark band in iodine chamber at 254 nm. In the HPLC analysis, single peak at Rt 4.3 min was observed in all the isolates used. Further this single peak can be characterized to know its toxin property. In the HPLC analysis of purified toxin *B.sorokiniana* showed a single peak at Rt 3.03 min with 96.16 purity and characterized as 'bipolaroxin' by H-NMR studies (Jahani *et al.,* 2013).

**Table 1: HPLC analysis of *B. oryzae* isolates**

| Isolates | Rt | Area | % Area | Height |
|---|---|---|---|---|
| BO 1 | 4.34 | 2528620 | 4.59 | 97736 |
| BO 2 | 4.15 | 6676871 | 13.34 | 102700 |
| BO 3 | 4.21 | 64617 | 0.60 | 4580 |
| BO 1319 | 4.26 | 512353 | 4.98 | 39601 |
| BO 5326 | 4.34 | 1007467 | 9.42 | 26424 |
| BO 23 | 4.22 | 766500 | 3.44 | 54070 |




Lane 1. BO 1; Lane 2. BO 2; Lane 3. BO 3; Lane 4. BO 5326; Lane 5. BO 1319 and Lane 6. BO 23

**Fig 1. Thin layer chromatography (TLC)**

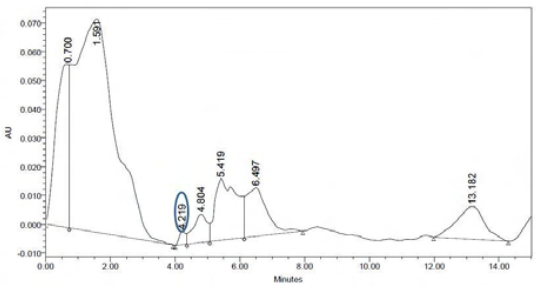



**Fig 2a.HPLC analysis of *B. oryzae* isolates (BO 1)**

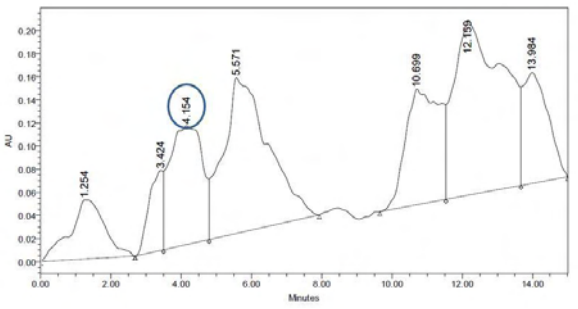



**Fig 2b.HPLC analysis of *B. oryzae* isolates (BO 2)**

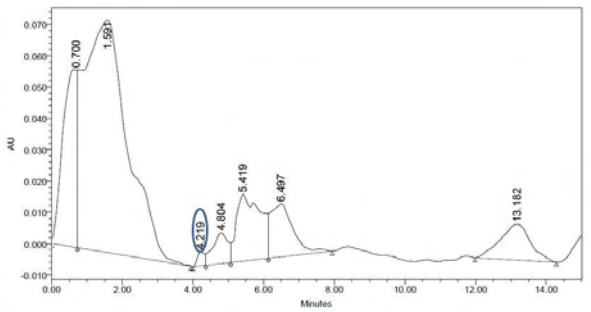



**Fig 2c.HPLC analysis of *B. oryzae* isolates (BO 3)**

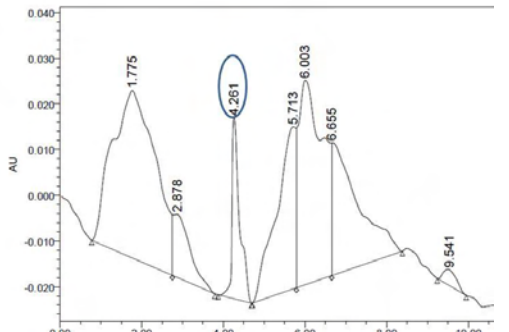



**Fig 2d.HPLC analysis of *B. oryzae* isolates (BO 1319)**

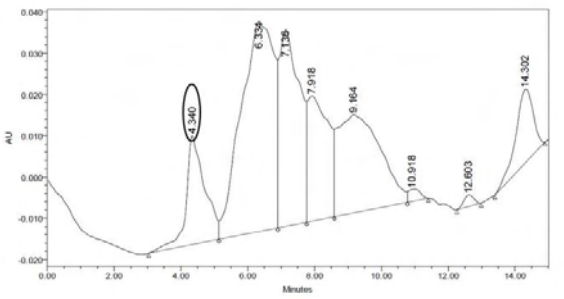



**Fig 2e.HPLC analysis of *B. oryzae* isolates (BO 5326)**

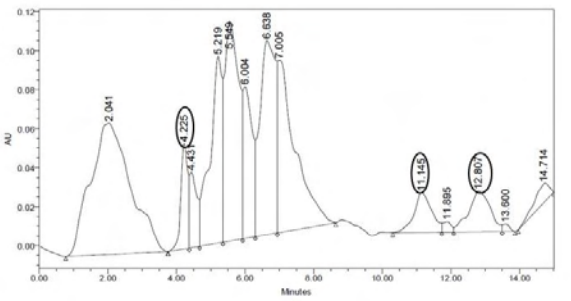



**Fig 2f.HPLC analysis of *B. oryzae* isolates (BO 23)**

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