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Qualitative and ecological impact of fungicide treatments for the management of aerial blight in soybean (*Glycine max* (L.) Merr.)

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Abstract
Aerial blight (RAB) is an important disease of soybean in the warm and humid regions of the world. This study analyses the impact of the different new generation fungicides and biocontrol agents used for the management of the disease on the qualitative parameters and to test the presence of their residues in the harvested soybean seeds. Results revealed that plots treated with strobilurin fungicides *viz.*, azoxystrobin and pyraclostrobin had enhanced oil and protein content, respectively. Among the bio-agents, *Trichoderma harzianum* treated plots recorded the highest oil content (20.50%); whereas the protein content was maximum in *Pseudomonas fluorescens* treated plots (37.77%). All the fungicides, except azoxystrobin (0.0071 ppm) and mycobutanil (0.21 ppm), was found to have their residue below the detection level and were safe for consumptions. Hence, these fungicides are safe from the qualitative and ecological point of view and can be recommended for the management of the Rhizoctonia aerial blight of soybean.

Keywords: Aerial blight, fungicides, qualitative assessment, residue, soybean

Introduction
Aerial blight of soybean caused by *Rhizoctonia solani* Kuhn (teleomorph: *Thanetophorus cucumeris* (Frank) Donk) is considered to be one of the most important foliar fungal disease of soybean worldwide. It occurs almost every year in severe form under favourable environmental conditions wherever soybean is cultivated. Soybean is infected by the pathogen at all stages of growth, which causes very rapid defoliation and frequent crop failure. Disease is wide spread in all soybean growing countries including areas of Mexico (Crispin and Gallegos, 1963) [5], all countries of central America and Caribbean islands (Echandi, 1966; Galindo, 1982) [8], Amazon region of Peru and Brazil (Muller, 1934; Deslandes, 1944) [31, 7], Colombia and Northwestern region of Argentina (Ploper, 1981) [32], etc. It has also been reported from United States, Japan, Philippines, Burma, Sri Lanka, Kenya and Malawi (Weber, 1939; Zaumeyer & Thomas, 1957; Mukunya, 1974) [39, 40, 30]. Mean soybean yield reduction of 35 per cent is attributed to aerial blight (Sinclair and Blackman, 1989) [33] with a potential of up to 70 per cent reduction (Hartman *et al.*, 1999) [17] under favourable environmental conditions. According to Fenille *et al.* (2002) [10], 31-60 per cent yield losses occur due to foliar blight of soybean in north and north east Brazil which under varying environmental conditions may range from 16-80 per cent. Up to 50 per cent loss in yield is reported from United States (Tachibana *et al.*, 1971; Joye 1986) [35, 21]. Aerial blight alone caused a yield loss of around three lakh tones of soybean during 2011 to 2014 in U. S. states and Ontario (Allen *et al.*, 2017) [2].

In India, the disease causes significant economic losses in the state of Uttarakhand, Chhatisgarh, Madhya Pradesh and Nagaland. Depending upon the environmental conditions, it can cause 40 to 50 percent yield loss (Mathpal and Singh, 2017; Joshi *et al.*, 2018) [27, 20]. The importance of the disease has increased due to the increased soybean production and expansion of soybean to new regions. The soil borne nature of the pathogen and its long term survival in the form of sclerotia makes it difficult to manage under field conditions (Manian and Manibhushanrao, 1990) [26]. With the impendent climate change, there is every possibility that the disease can occur in epidemic form in the years to come (Singh *et al.*, 2019) [34]. Since an immune/completely resistant commercial variety is lacking, farmers mainly depend on the chemical fungicides and few biocontrol agents for the management of this dreadful disease.

The use of fungicides is being limited due to the widespread occurrence of the harmful residue and possible impact on the nutritional and qualitative parameters in the harvested produce. The new generation fungicides, like the strobilurins and triazoles, are advocated to be ecologically safer than the conventional chemical fungicides as they are target specific and used in lower amount with high efficiency.

Since the qualitative and ecological impact of these fungicides in soybean is not well documented, the present study was under taken to test the residue status and impact of these fungicides on the nutritional parameters of soybean.

Materials and Methods

A field experiment was laid out in randomized block design at Norman Borlaug Crop Research Centre, G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand for three consecutive crop seasons from 2015 to 2017 to test the efficacy of twelve fungicides and three bio-control agents for the management of Rhizoctonia aerial blight of soybean. The plots without the treatment served as control. Seeds of a highly susceptible cv. JS-335 were sown in plots (4x1.8 m^2). The experiment was laid out in randomized block design with three replications. All the recommended agronomic practices were carried out.

Qualitative assessment

Qualitative assessment of field treatments was done by quantifying total oil and protein content in harvested soybean seeds.

Total oil content

Total oil content in the soybean seeds was assessed using the soxhlet assembly (Jensen, 2007) [19]. Two grams of the finely grinded soybean samples were packed in Whatman filter paper no.1 and the final weight was again taken. 150 mL of ethane was poured into 250 mL round-bottomed flask in soxhlet assembly. After six hours of extraction, the solvent was removed and the samples were dried overnight. The final weight of the dried samples was taken and the oil content in the samples was calculated using the following formula:

Total oil content (%) = $[(W_2 – W_3)/ (W_2 – W_1)] \times 100$

Where,

W_1 – Weight of the filter paper used

W_2 – Initial weight (filter paper + sample)

W_3 – Final weight after oil extraction (filter paper + sample)

Total protein content

Total protein content in the soybean seeds was assessed using the Bradford method (Bradford, 1976) [3]. Soybean seeds were homogenized in protein extraction buffer and samples were prepared by taking 20 µL of soybean seed extract in 80 µL of autoclaved distilled water and 3 mL of Bradford dye. Sample tubes were then incubated for 30 min and the absorbance was measured at 595 nm using a spectrophotometer. The total protein content was estimated using the standard graph plotted using the absorbance of bovine serum albumin standards.

Protein quality using SDS page

The extracted protein samples from the seeds of soybean were analyzed by SDS-PAGE using the method described by Laemmli (1970) [22]. The vertical slab gel electrophoresis apparatus (Genei, India) having 12 percent resolving gel and 5 percent stacking gel was used for performing SDS-PAGE.

Ecological assessment of field treatments

To assess the ecological impact, residue analysis of the chemicals used in the field experiment was done in the harvested soybean seeds. Due to unavailability of standards, only nine of the twelve chemical fungicides could be evaluated for the residue content. Samples were sent to the Institute of Pesticide Formulation and Technology, a central government undertaking, in the Department of Chemicals and Petrochemicals under Ministry of Chemicals and Fertilizers, Gurgaon. The soybean seed samples were finely grinded and five gram sample was weighed in 50 mL centrifuge tube. Ten milliliter distilled water, 15 mL CAN, 150 µL acetic acid, 6 g anhydrous Mg SO_4 and 1.5 g NaCl were added. The mixture was vortexed for 1 min and further centrifuged for 5 min at 4000 rpm. Six ml of supernatant solution was transferred to a centrifuge tube and 900 mg $MgSO_4$ (anhydrous), 300 g PSA and 150 mg C18 were added followed by centrifugation for 5 min at 400 rpm. Three mL supernatant solution was taken from the above mixture, evaporated to dryness and reconstituted with 500 ml n-hexane (for GC-MS)/ ACN (for HPLC)/ Methanol (for LC-MS/MS).

GC-MS

In GC-MS, the MS system used was of 5975C EI/MSD with triple axis detector and GC system was of Agilent technologies, 7890A. Capillary column of 35 mm length was used in the analysis.

HPLC

For conducting the residue analysis by HPLC, Dionex (PDA detector) having multichannel wavelength ranging from 200 nm to 800 nm (Model no. Ultimate 3000) was used. The solvent system was acetonitrile (75%) + water (25%) with a solvent flow rate of 1mL /min. Brownlee analytical phenyl column of dimensions 5 µm, 150 x 4.6 mm was used and the fungicides were tested at 225 nm wavelength.

LC-MS/MS

In LC-MS/MS, MS system used was of Agilent 6410A Triple Quadrupole. The run time was 5 min and single reaction monitoring was done. Two solvent phases *viz.* methanol and water were used. Gradient flow was methanol (10%) + water (90%) for 2.5 min reverse and later methanol (90%) + water (10%) till the end. 0.1 percent formic acid and 1 mM ammonium formate buffer was used for the analysis. Multi residue method with flow rate of 0.4 mL /min was employed. RP18 (reverse phase), thermo scientific, 5 µm particle size column with 38 mm length was used.

Results

Oil quantification

The results revealed that all the treatments were at par with the untreated control plot (Table 1). The oil content in all the treatments ranged from 18.21 to 21.60 percent. Maximum oil content was recorded in soybean plants treated with azoxystrobin (21.60%) followed by pyraclostrobin (21.39%) and difenconazole (21.06%). The minimum oil content was observed in myclobutanil (18.45%) followed by dimethomorph (18.98%) treated plots. Other fungicides exhibited moderate increase in oil content of soybean seeds. Bioagents were found to have more positive effect on oil content compared to many chemical fungicides with *T. harzianum* and *B. subtilis* having 20.50 and 20.28 percent oil content, respectively.

Table 1: Oil and protein content of soybean seeds harvested after foliar spray of fungicides and antagonists for the management of Rhizoctonia aerial blight

Treatment	Oil (%)	Protein (%)
Triadimefon	19.56	37.43
Chorothalonil	20.51	37.45
Tricyclazole	19.61	37.76
Pyraclostrobin	21.39	37.95
Myclobutanil	18.45	37.95
Dimethomorph	18.98	37.80
Hexaconazole	19.30	37.79
Fluopyram	19.77	37.43
Difenoconazole	21.06	37.57
Azoxystrobin	21.60	37.54
Pencycuron	19.56	37.47
Tebuconazole	19.34	37.48
T.harzianum	20.50	37.63
P. fluorescens	19.43	37.77
B. subtilis	20.28	37.58
Control	18.21	37.37
CD (p=0.05)	0.18	0.36
CV	0.55	0.57

Protein quantification

The studies on the effect of fungicide treatment on protein content of the soybean seeds revealed that there was no significant change in the protein content of the seeds due to fungicide application. The protein content in treated plots varied between 37.43-37.95 percent (Table 1). Highest protein content was recorded in pyraclostrobin (37.95%) and myclobutanil (37.95%) during the present investigations. Protein content in tricylcazole (37.76%), dimethomorph (37.80%), hexaconazole (37.79 %) and *P. fluorescence* (37.77%) treated plots were higher than untreated control while rest of the treatments protein content were at par. It could be clearly inferred from the above data that fungicide and antagonist spray had little effect on the protein content of the soybean seeds.

Quality of protein

The SDS-PAGE protein profiling results of the soybean seeds obtained after application of different fungicides and bio agents are presented in Figure 1. The results indicated no major differences in the quality of protein in the treated plots when compared to control. The banding pattern obtained in different treatments was similar in all treatments. The intensity of protein bands was only slightly variable in different treatments. Hence, a more sophisticated techniques need to be adopted to evaluate minor changes at individual protein level.

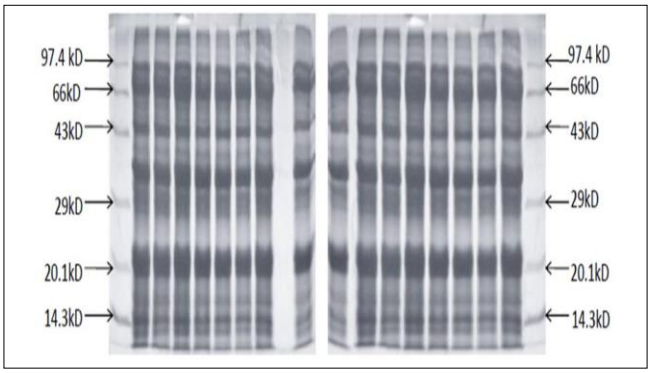

Fig 1: SDS profile of proteins isolated from different soybean seeds treated with fungicides and antagonists.

Lane1: ladder, Lane2: triadimefone, Lane3: chlorothalonil, Lane4: tricyclazole, Lane5: pyraclostrobin, Lane6: myclobutanil, Lane7: dimethomorph, Lane9: hexaconazole, Lane10: fluopyram, Lane11: difenoconazole, Lane12: azoxystrobin, Lane13: pencycuron, Lane14: tebuconazole, Lane15: *T. harzianum*, Lane16: *P. fluorescens*, Lane 17: *B. subtilis*, Lane 18: control, Lane 19: ladder.

Ecological effect of field treatments of soybean

In the present study, average recovery of all the fungicides was 85 percent (Table 2). All the samples were tested using GC-MS except difenconazole and azoxystrobin which were analyzed using HPLC and LC-MS/MS, respectively. The results of the residue analysis of individual chemical pesticides have been discussed under.

Table 2: Residue limit of fungicides as detected for different chemical fungicides used for the management of *R. solani* causing Rhizoctonia aerial blight

Treatment	Limit of detection (LOD)	Limit of Quantification (LOQ)	Residue (ppm)	Method
Triadimefon (detected as Triadimenol)	0.033 mg/kg	0.1 mg/kg	BDL[a]	GC-MS
Chorothalonil	0.033 mg/kg	0.1 mg/kg	BDL	GC-MS
Tricyclazole	0.033 mg/kg	0.1 mg/kg	BDL	GC-MS
Myclobutanil	0.033 mg/kg	0.1 mg/kg	0.21	GC-MS
Dimethomorph	0.033 mg/kg	0.1 mg/kg	BDL	GC-MS
Hexaconazole	0.033 mg/kg	0.1 mg/kg	BDL	GC-MS
Difenoconazole	0.033 mg/kg	0.1 mg/kg	BDL	HPLC
Azoxystrobin	104 ppt	312 ppt	0.0071	LC-MS/MS
Tebuconazole	0.033 mg/kg	0.1 mg/kg	BDL	GC-MS

a) Triadimefon: It was analysed through GC-MS method. It was observed that in the plant system, it got converted into triadimenol, which indicates that the chemical has transferred itself from the ketone to alcohol form (Table 2, Supplementary Figure 1). The residue of triadimenol was found below the detection limit of the equipment. According to Ministry of Health, Labour and Welfare Japan (2006) [29], residue limit for triadimenol in soybean is 0.2 mg/kg which is higher than the detection limit of the instrument.

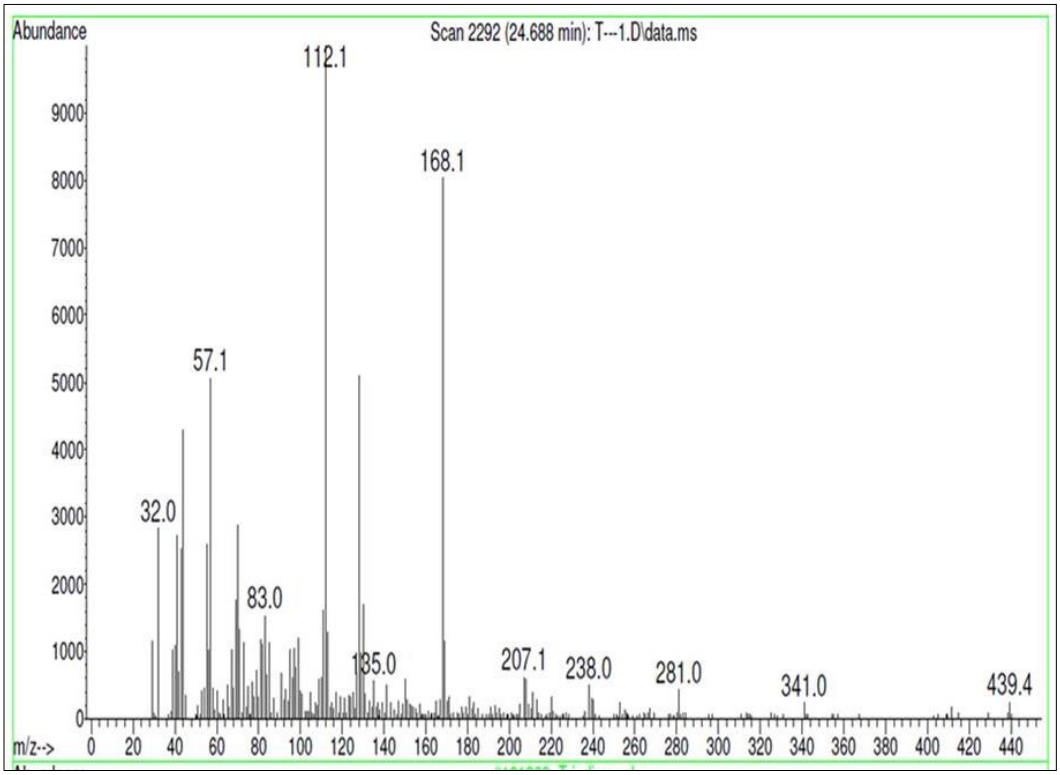

Fig 2: MS spectra of soybean seeds harvested after field treatment with triadimefon (analysed as triadimenol).

b) Chorothalonil: The results (Table 2, Supplementary Figure 2) revealed that the compound could not be detected, implying the residue to be below the detection limit of the instrument. The results were in accordance with the findings of US Environmental Protection Agency Office of Pesticide Programs pesticide (2012) [38] were upper pesticide limit of cholorothalonil in soybean grain is 0.2 ppm and Food Safety and Standards Authority of India (2011) [11] were the above chemical is registered in groundnut with an MRL of 0.10 ppm.

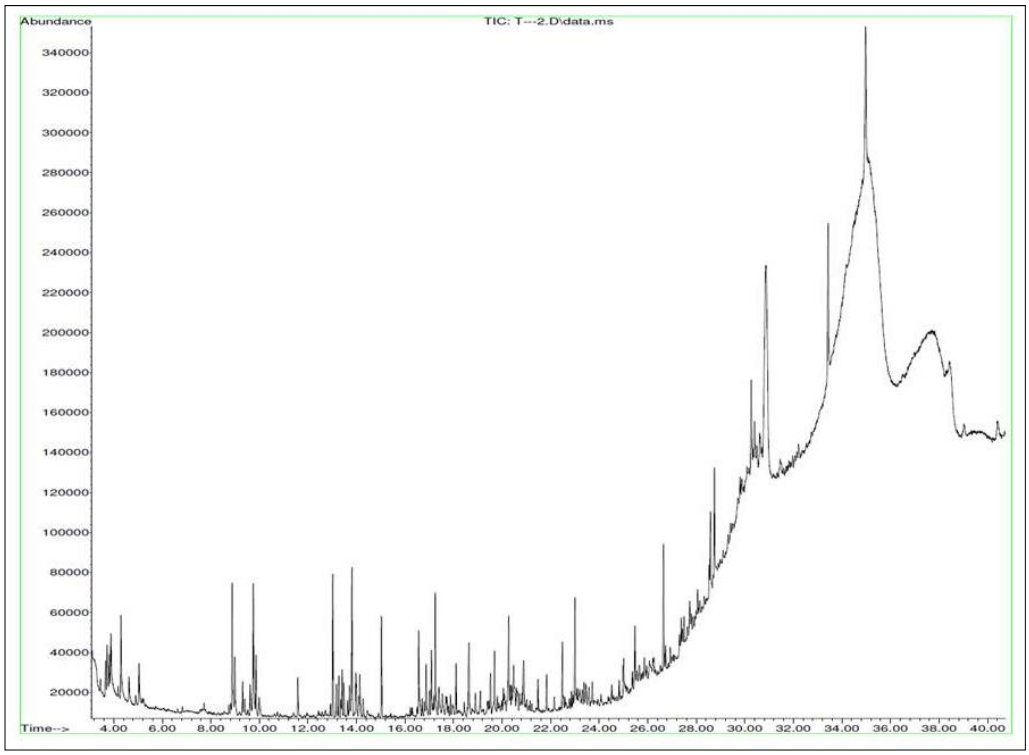

Fig 3: Chromatogram of soybean seeds harvested after field treatment with chlorothalonil

c) Tricyclazole: It was analysed through GC-MS method, and the chromatogram (Table 2, Supplementary Figure 3) of tricyclazole revealed the absence of the desired peak indicating that it is below the detection limit of the instrument. As per The Japan food chemical research foundation (2016) [36] database, MRL limit of tricyclazole in oil seeds is 0.02 ppm, which is more than the obtained value.

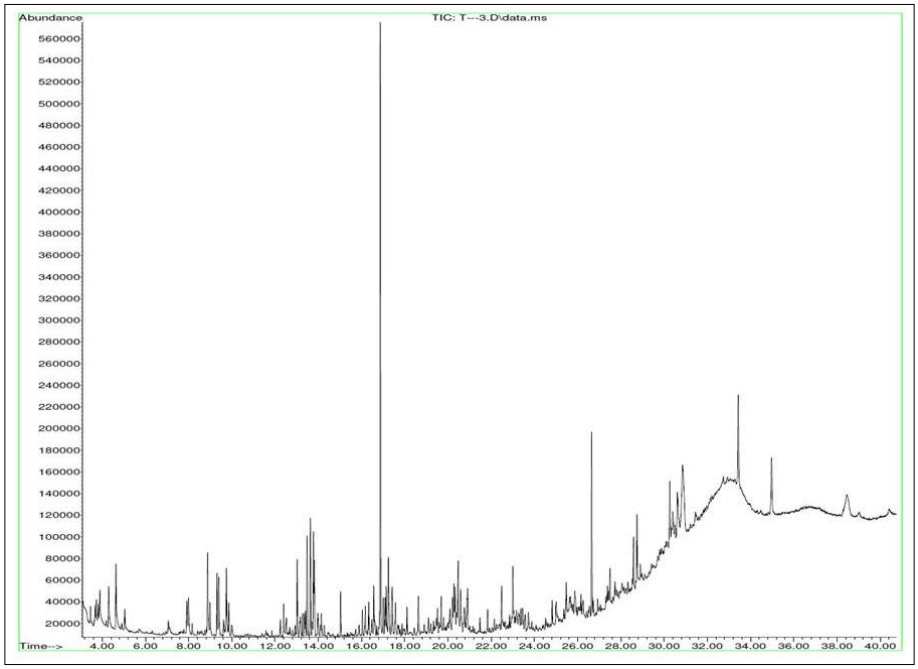

Fig 4: Chromatogram of soybean seeds harvested after field treatment with tricyclazole.

d) Myclobutanil: The results of the analysis of the residue of myclobutanil in soybean seeds through GC-MS method revealed that the compound was present in appreciable amount (0.21 ppm) *i.e.* above the detection limit of the equipment. Table 2 and Figure 2 reveal the chromatogram of the myclobutanil treated soybean samples. The presence of the desired peak at 25.87 min corresponding to myclobutanil confirms the presence of myclobutanil in the seed samples. The results were in accordance with the set MRLs of Codex Alimentarious Committee (2015) which is 0.8 mg/kg for beans and US Environmental Protection Agency Office of Pesticide Programs pesticide (2012) [38] were upper pesticide limit of cholorothalonil in soybean is 3 ppm.

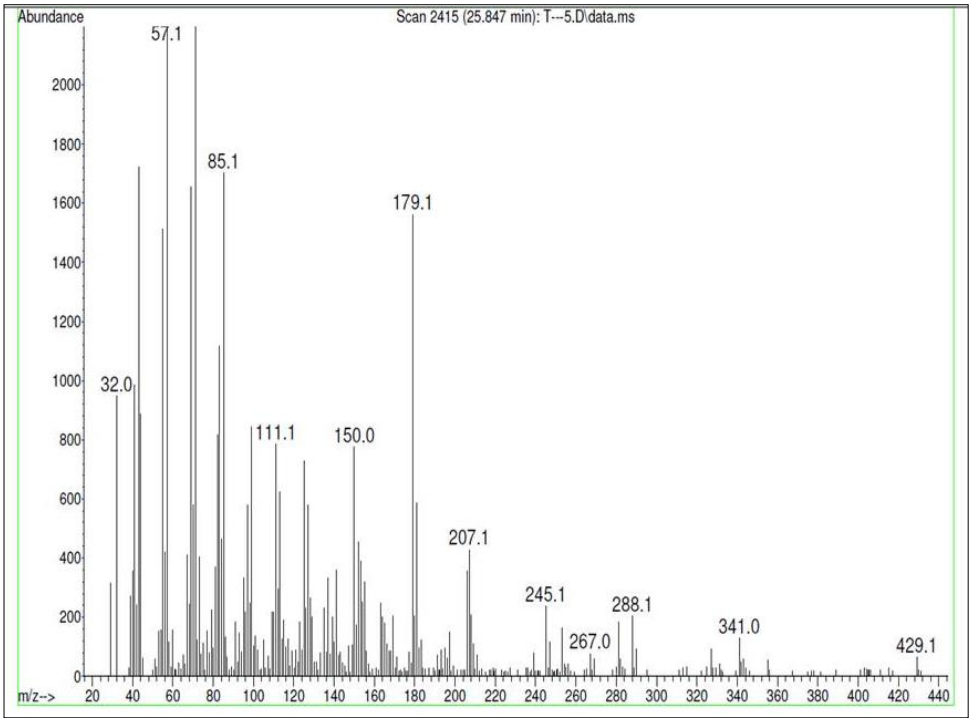

Fig 5: MS spectra of soybean seeds harvested after field treatment with myclobutanil.

e) Dimethomorph: It was analysed through GC-MS method, and the chromatogram (Table 2, Supplementary Figure 4) of dimethomorph revealed the absence of the desired peak indicating the absence of the compound and thus being below the detection limit of the GC-MS unit. The results were in accordance with the set MRLs of Codex Alimentarious Committee (2015) which is 0.7 mg/kg for beans which is far above than the limit of detection of the instrument.

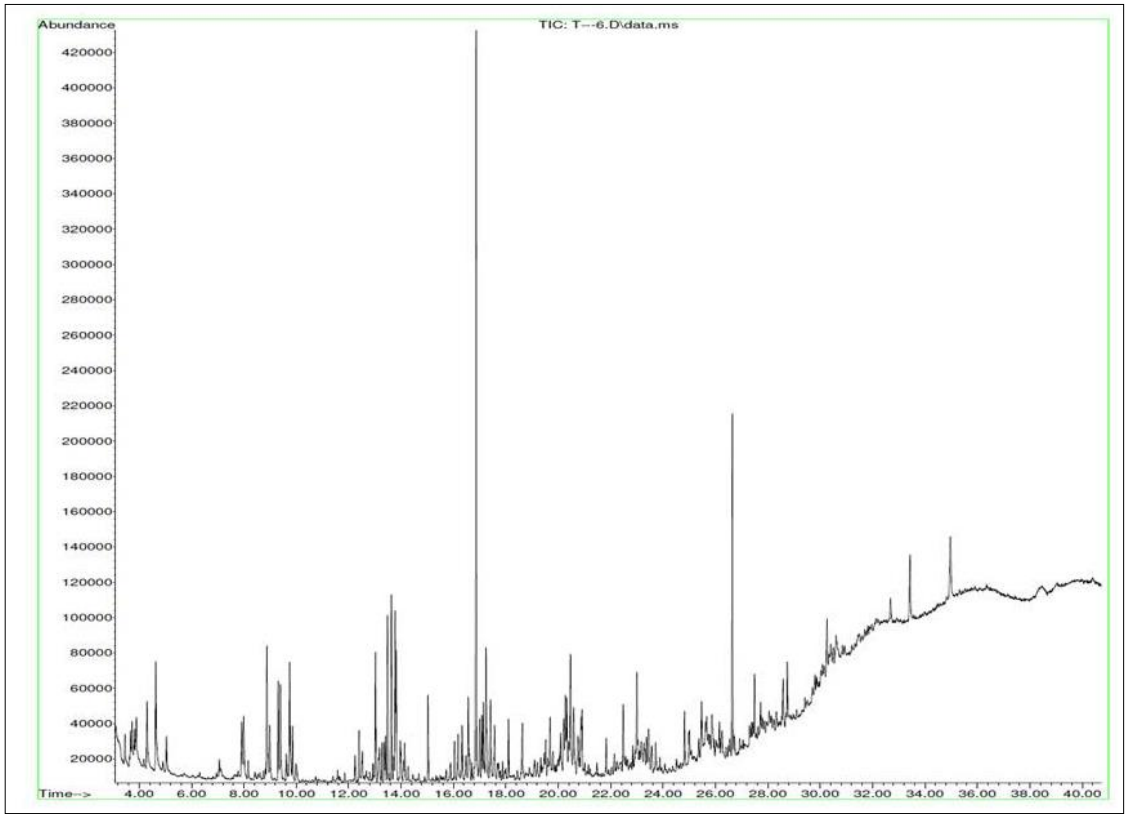

Fig 6: Chromatogram of soybean seeds harvested after field treatment with dimethomorph

f) Hexaconazole: The result of the analyses of hexaconazole through GC-MS method (Table 2, Supplementary Figure 5) indicate the absence of the desired peak indicating the absence the compound and thus being below the detection limit of the GC-MS unit. The MRL limit of hexaconazole in oil seeds set by The Japan Food Chemical Research Foundation (2016) [36] database is 0.05 ppm, which is above the limit of detection implying the commodity to be fit for consumption.

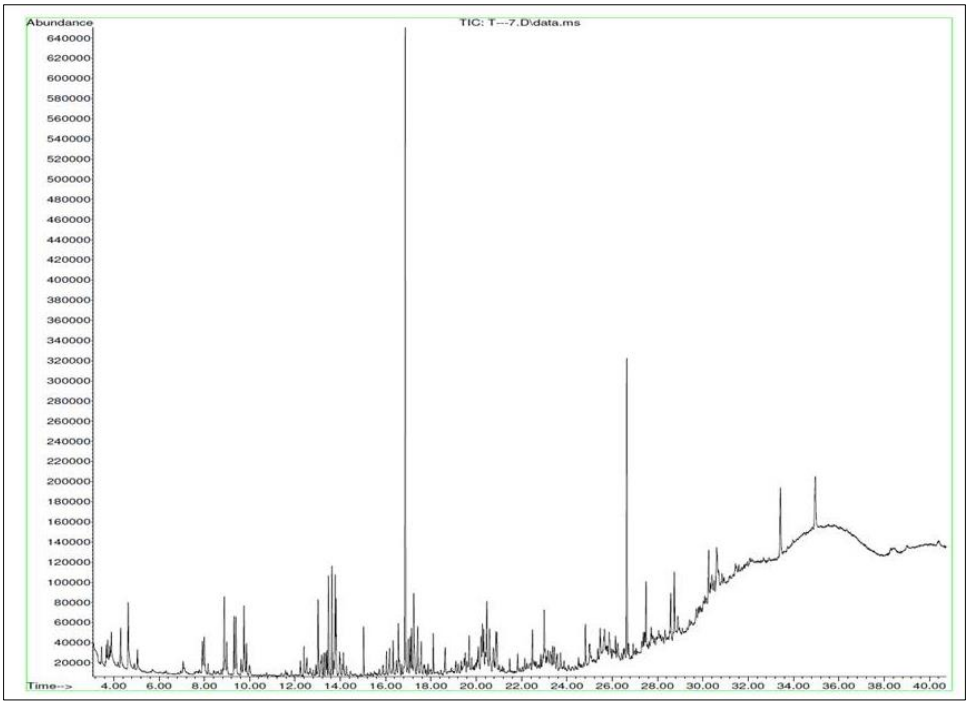

Fig 7: MS spectra of soybean seeds harvested after field treatment with hexaconazole.

g) Difenoconazole: The difenoconazole treated soybean seed samples were analysed through HPLC method. Table 2 and Supplementary Figure 6 indicate that the compound could not be detected. Thus it can be concluded that the compound was present below the detection limit of the HPLC unit. The results were in accordance with the set MRLs of difenoconazole by Codex Alimentarious Committee (2015) (0.7 mg/kg for beans) and The Japan Food Chemical Research Foundation (2016) [36] (0.05 ppm for soybean).

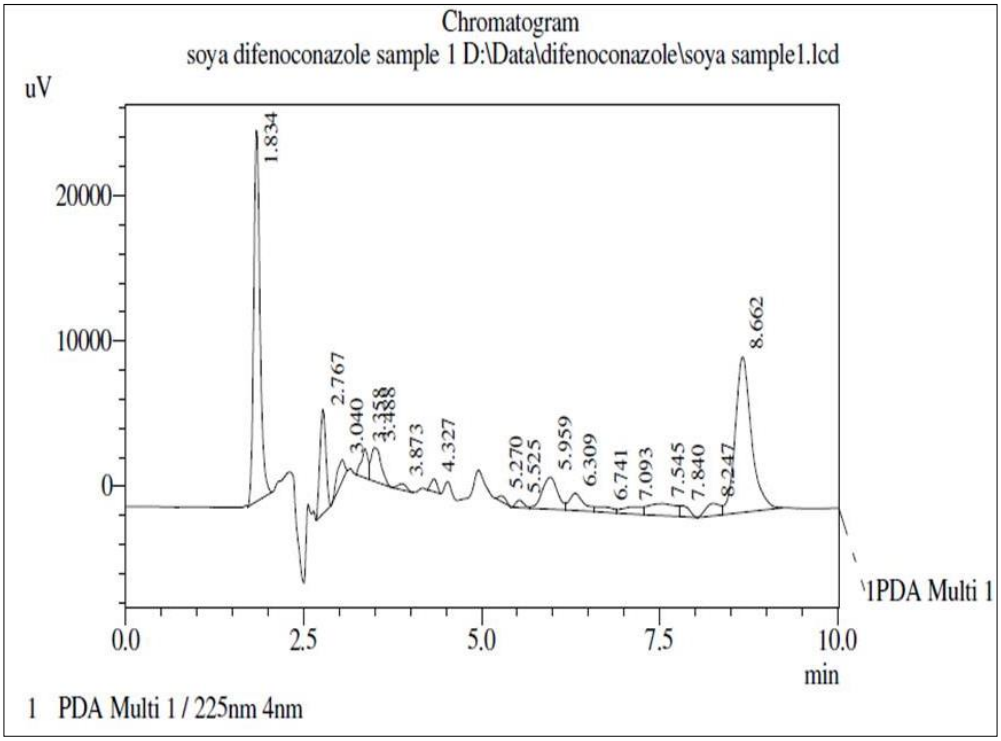

Fig 8: Chromatogram of soybean seeds harvested after field treatment with difenconazole.

h) Azoxystrobin: The azoxystrobin treated soybean samples were analysed with more sophisticated method of LC-MS/MS. The chromatogram (Table 2, Figure 3) results indicate the azoxystrobin peak at 3.4 min. The quantity of azoxystrobin detected was 0.0071 ppm. The results are in accordance with the set MRL limits of Codex Alimentarious Committee (2013) for soybean were the residue limit for azoxystrobin is 0.5 ppm and the commodity is thus safe for consumption.

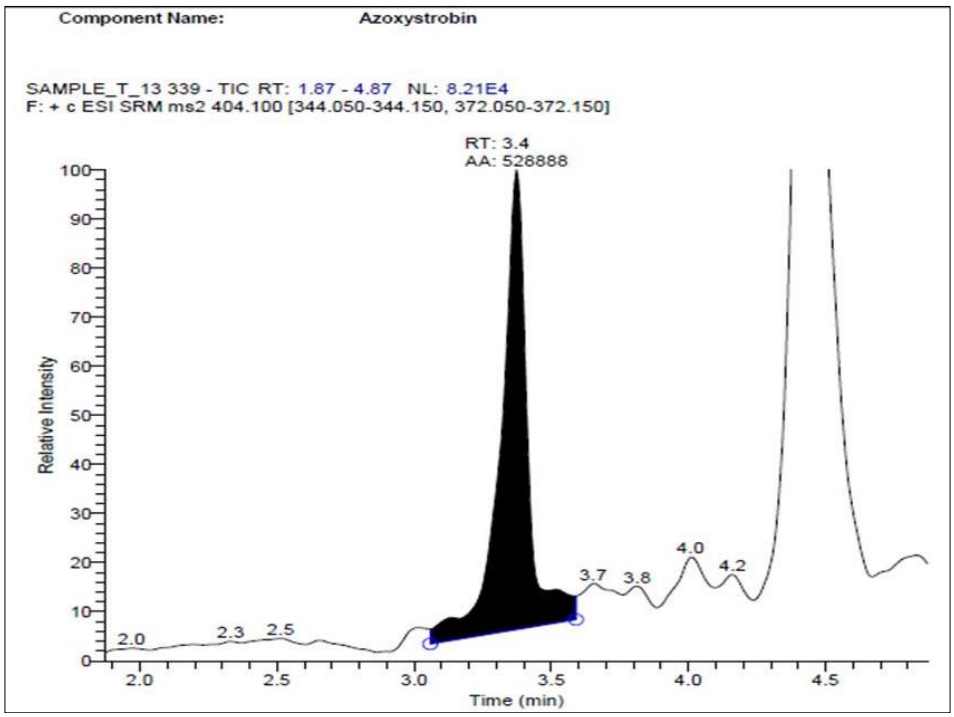

Fig 9: Chromatagram of soybean sample harvested after field treatment with azoxystrobin.

i) Tebuconazole: The result of the analyses of tebuconazole through GC-MS method presented in Table 2 and Supplimentary Figure 7 reveals the absence of the desired peak indicating the residue level below the detection limit of the GC-MS unit. The results were in accordance with the set MRLs of Codex Alimentarious Committee (2015) which is 0.3 mg/kg for beans and US Environmental Protection Agency Office of Pesticide Programs (2012) [38] were upper pesticide limit of tebuconazaole is 5 ppm.

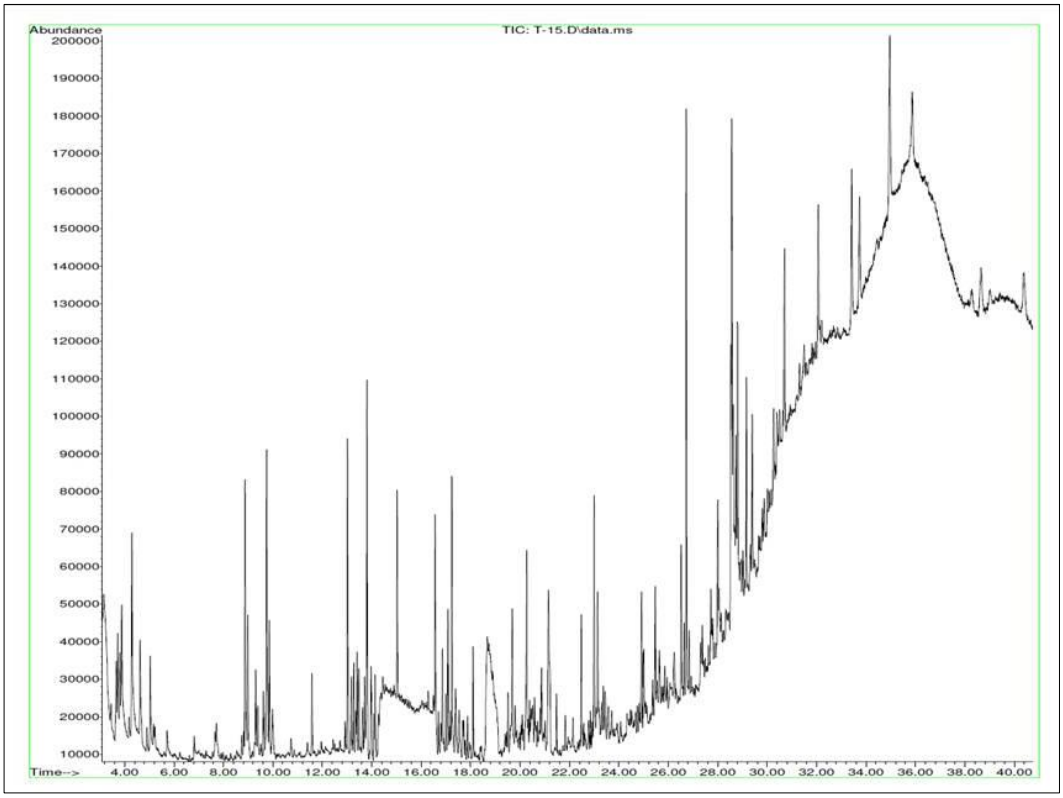

Fig 10: Chromatogram of soybean seeds harvested after field treatment with tebuconazole

Discussion

The findings of the experiment clearly demonstrate that none of the fungicides or the biocontrol agents used to manage the Rhizoctonia aerial blight of soybean had any negative impact on the qualitative parameters of soybean and are ecologically safe as well. Both the fungicides and biocontrol agents were found to improve the oil and protein content as compared to the untreated control. Oil content was increased by as much as 18.61 percent in the azoxystrobin treatment when compared to that of control. Interestingly, it was observed that, fungicides belonging to strobilurin group have significantly higher impact on oil content as compared to others. The plots receiving the treatment with biocontrol agents showed an average oil content increase of 10. 21 percent, with the maximum increase reported in *T. harzianum*. *Trichoderma harzianum* was found to have maximum effect on disease suppression as well. Our findings are consistent with those from previous studies in soybean and other related crops. The application of the fungicide ApronMaxx® (mefenoxam + fludioxonil) was found to have a positive impact on the oil and protein content in soybean (Lenssen, 2013) [23]. McCartney *et al.* (2016) [28] have observed that in oilseed rape, fungicide treated plots tend to have higher oil yields even with low disease levels, implying independent physiological effect of disease. Turk *et al.*, (2008) [37] and Butkute *et al.*, (2006) [4] have also reported that oil content of rapeseed increased significantly after application of triazole fungicides.

Similarly, all the tested treatments have found to enhance the protein content in soybean seeds. The protein content ranged from 37.43 to 37.95 percent in case of fungicides and 37.58 to 37.77 percent in case of biocontrol agents. This implies that both the fungicides and biocontrol agents have a positive impact on the nutritional qualities soybean. The findings of our study are in accordance with that of previously published literatures. Abbasian *et al.* (2012) [1] have reported that the application of fungicides modified the grain protein content in sunflower. The combined application of strobilurin fungicide and triazole fungicides were found to have a positive impact on the oil and protein content of winter rape seed. The application of Toprex® (Difenconazole + paclobutrazol) along with Ortiva® (Azoxystrobin) increased the oil, free fatty acid and protein content by 1.5, 0.2 and 0.8 percent, respectively (Ijaz *et al.*, 2015) [18]. The increase in nutritional parameters may be attributed to enhancement of seed health by reducing disease pressure and physiological impact of certain fungicides. According to Hanumantharaju *et al.* (2010) [16], the bioagents have increased protein content in soybean cv. JS-335 when applied in integration with *Rhizobium* and/ or arbuscular mycorrhizae. Thus, it can be inferred that, bioagents helped in uptake and mobilization of nitrogen resulting in synthesis of more protein.

Seed samples treated with mycobutanil and azoxystrobin were found to have 0.21 ppm and 0.0071 ppm residue level, respectively; while the other fungicides tested were found to be below the permissible minimum residue limit in the soybean seeds. In many cases, there are no MRL limits set for the described chemicals in India. For such fungicides, MRLs of other countries have been taken as reference. Results indicate that these fungicides can be used for managing *R solani* fungi under field conditions as the residues of these fungicides at harvest were lower than the set international standards of MRL indicating them to be safe to be used for human and animal consumption. In our study, we also observed that some of the applied fungicides gets modified/ converted into a new form within the plant system. For example, the fungicide triademefone was found to get converted into triademenol in soybean. Similar enantioselective degradation has been reported by many scientists (Deas *et al.*, 1984; Garrison *et al.*, 2011; Liang *et al.*, 2013; Li *et al.*, 2014) [6, 14, 25, 24]. This degradation is dependent on environmental conditions, type of microorganism present in the environment and host plant species which further affects the systemic fungitoxic effect **(**Gasztonyi and Josepovits, 1979) [15]. Hence, during the

analysis for the residue in the final product, we need to consider these aspects as well.

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