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Effect of bio-inoculation on physical health of cabbage seedlings and disease dynamics of Alternaria leaf spot in cabbage under challenge inoculation

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Abstract
Cabbage is one of the important vegetable crops grown in India. India being the second largest producer of vegetables accounted for 12% of the total cabbage production in the world for the year 2012-2013 [8]. Alternaria leaf spot caused by *Alternaria sp. (A. brassicae and A. brassicicola)* has been reported from all the continents of the world and is one among the common diseases of cabbage. Alternaria blight is one of the most dominant one that causes average yield loss in the range of 32-57% [5]. Bio-inoculated seedlings exhibited significantly higher vigour index. Seedling vigour was found to be increased in single or combination of *Trichoderma* and *Pseudomonas fluorescence* applied through seed treatment. Higher root and shoot dry biomass observed when *Trichoderma* applied as seed treatment. The rate of chlorophyll degradation was less in two treatments *P. fluorescence* applied through seed treatment and combined inoculation of *Trichoderma* and *P. fluorescence* applied through seed treatment. Low AUDPC was recorded, were *Trichoderma* and *P. fluorescence* applied combined through seed treatment.

Keywords: Alternaria leafspot, *Trichoderma*, *P. fluorescence*, Vigour index, AUDPC

Introduction
Cabbage is one of the most popular winter vegetable grown in India. India is one of the important cabbages growing country in Asia. India is next to China in cabbage production [4]. The area under cabbage production for the year 2012-13 is 372 million hectares with a production of 8534 Million tones and a yield of 22940.9 Kg/hectare respectively.
Besides, good technology and certified seeds, the desirable production is not achieved because of damages caused by insect pests and diseases. In India about 20% of the crop yield is lost due to insect pests and disease per year, which approximately amount to Rs. 1500 crores but in case of outbreaks, loses, increased upto 50-90% [18]. Alternaria black leaf spot disease is one the most destructive disease of cabbage and brassicas worldwide [14]. A complex of *Alternaria species* (*A. brassicicola* (Schw.) Wiltsh., *A. brassicae* (Berk.) Sacc., *A. alternata* (Fr.) Kreissler and *A. raphani* Groves and S kolko) are responsible for considerable yield losses [21]. The pathogens are greatly influenced by weather with the highest disease incidence reported in mild, wet seasons and in areas with relatively high rainfall [11]. The pathogen appears on leaves and stems of cabbage seedlings and adult plants. It can also affect the siliquae causing a severe reduction in the amount and the quality of head or seed production. Cabbage can be affected in all stages of growth, thus typical symptoms include black necrotic lesions surrounded by chlorotic areas on leaves, stems and siliquae [13].
A. brassicae and *A. brassicicola* can affect host species at all stages of growth, including seeds. On seedlings symptoms include dark stem lesions immediately after germination, that result in damping-off, or stunted seedlings [20]. In addition to destruction of a seed crop, the pathogens can live within the seed, spread the disease to other fields, and cause a loss of seedlings [15].

Materials and methods
Bio-inoculation of *Trichoderma sp* and *Pseudomonas fluorescence* to identify the Effect of bio-inoculation on physical health of cabbage seedlings under pot condition were tested during 2016-17 rabi crop season at the Department of Plant Pathology (Uttar Banga Krishi Viswavidyalaya Cooch Behar). Five treatments viz, seed treatment, soil application, seed treatment + soil application, seed treatment + foliar application and foliar application by using *Trichoderma harzianum*, *Pseudomonas fluorescence* and *Trichoderma harzianum* + *Pseudomonas fluorescence.*

Bio agents
Pure culture of *Trichoderma harzianum* (UBT18) and *Pseudomonas fluorescence* (VPF-1) was obtained from Biocontrol Laboratory, Department of Plant Pathology, UBKV, Cooch Behar.
Pseudomonas fluorescence strain VPF-1 was mass multiplied on King´s B broth IN 250 ML Erlenmeyer flasks, incubated at 28 ºC for 48 hrs. in shaker incubator. Cell suspension of bacterial strain was adjusted to concentration of 10^6 CFU/ml and used along with talc and Carboxy methyl cellulose. *Trichoderma harzianum* (UBT18) was mass multiplied on potato dextrose broth, after full mycelia growth obtained media mixed with talc and Carboxy methyl cellulose.

Preparation of pathogen inoculums
Alternaria sp. was isolated from leaf spot affected cabbage leaf. The fungal inoculums were taken from petridishes and transferred to potato dextrose broth (PDB) and was kept for 7 days of incubation. After the full growth of pathogen the mycelial mat was harvested and homogenized. Conidial concentration was determined using a haemocytometer and adjusted to 5 x 10^5 conidia per mL. Plants were labeled and spray inoculated with a conidial suspension of pathogen using 1000 mL hand held atomizer directed at the central part of the upper leaf side. Approximately 0.3 mL conidial suspension per leaf was applied to the plants.

Mode of application
Seeds were treated with *Trichoderma harzianum* and *Pseudomonas fluorescence* at the rate of 5 gram per kg, of seed, applied 30 min. before sowing. *Trichoderma harzianum* and *Pseudomonas fluorescence* were applied in soil at the rate of 12.5 kg per ha. Foliar spray of *Trichoderma harzianum* and *Pseudomonas fluorescence*, at 1% concentration of talc based formulation done 5 days before pathogen inoculation.

Plant growth promotion
Plant growth promotion activity of *Trichoderma harzianum* and *Pseudomonas fluorescence* were assessed based on the seedling vigour index was calculated by using the formula described by Abdul Baki and Anderson [1]
Vigour Index = mean root length + mean shoot length x germination (%)

Chlorophyll estimation
Chlorophyll content estimated by using Konica Minolta SPAD-502 PLUS at transplanting stage and before challenge inoculation

Shoot and root dry weight
Seedlings at the transplanting stage kept at hot air over for 7 days and measured the shoot and root dry biomass.

Percent disease index
Percent disease index (PDI) was calculated for each plot by summing the scores of twenty leaves and analyzing using rating scale. The value was expressed as percentage using the following formula:

$$PDI\ (\%) = \frac{\text{Sum of all rating} \times 100}{\text{No. of leaves examined x maximum rating score}}$$

No. of leaves examined x maximum rating score
Scale (0-6) used for rating:
Rating Symptoms of Alternaria blight on leaves[16]
0=No infection
1=Up to 5% leaf area covered
3= 5-10% leaf area covered
5=11-25% leaf area covered
7= 26-50% leaf area covered
9 =More than 50% leaf area covered

AUDPC
Area under Disease Progress Curve (AUDPC) was calculated by using the following formula [23]

$$AUDPC \sum_{i-1}^{k} 1/2\ (S_i - S_{i-1})\ d$$

Si = Disease severity at the end of time I, k = Number of successive evaluation of blight severity
d = Interval between two observations

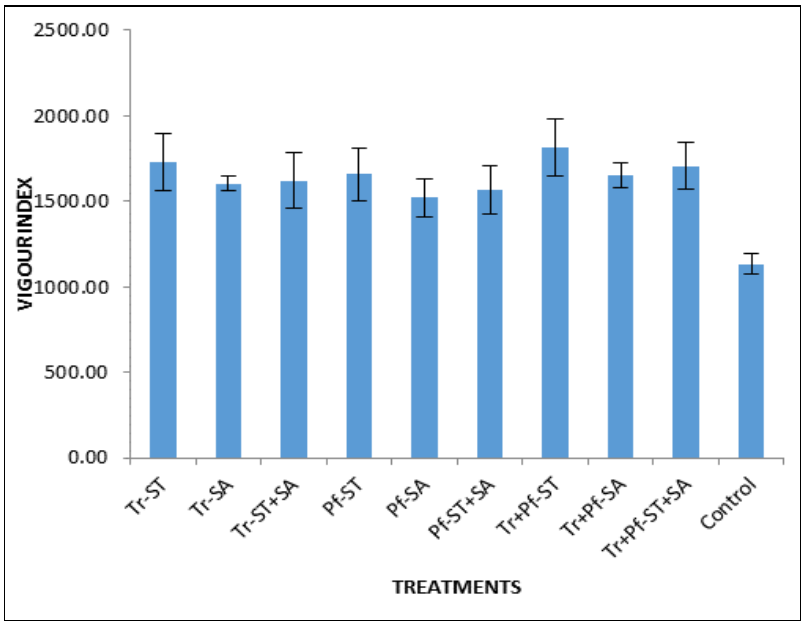

Fig 1: Effect of bio-inoculation on vigour index of cabbage seedlings

Result and Discussion

Effect on Vigour Index

Vigour index is one of the most important indices represents the plant health. In the present investigation the results on vigour index (Fig.1) revealed that irrespective of delivery system i.e. through seed treatment or soil application or combination of both bio-inoculated seedlings add significantly higher vigour index (1520.00-1813.33) in comparison to untreated check, on the other hand single or combination of *T. harzianum* and *P. fluorescence* applied through seed treatment resulted comparatively higher vigour index. Seed treatment along with soil application did not provided better results as compared to seed treatment alone. Although many research findings reported that seed treatment is the most suitable way to introduce biocontrol agents to the spermosphere rather than soil application but however seed along with soil application also exhibit incomplete rhizhosphere colonization which reflects through higher vigour index but it may not always be true due to spatial structure of population leading to repeated interaction between the mutualists [9].

Results on dry biomass of root and shoot of cabbage seedlings at transplanting stage have been presented in table 1 which revealed that shoot and root dry biomass increase significantly over control. Significantly higher shoot biomass was observed when *Trichoderma* was applied thorough seed treatment (0.075 mg/seedling) which was significantly at par with *Trichoderma* applied through seed treatment and soil application, *P. fluorescence* through seed treatment and soil application and combined application of *Trichoderma* and *P. fluorescence* seed treatment (0.05-0.069 mg/seedling). Later three was only significantly at par with *Trichoderma* applied through soil application and combined inoculation of *Trichoderma* and *P. fluorescence* through seed treatment and soil application (0.060 and 0.059 mg/ seedling respectively). The result was also in corroboration with the previous results of vigour index, seed treatment was comparatively better than applied through seed treatment and soil application in combination. Root dry weight also follow almost the same trend, were *Trichoderma* through seed treatment resulted significantly higher root dry biomass (0.016 m/seedling) and it was significantly at par with *Trichoderma* application with seed and soil (0.014 mg/seedling). The increase in shoot and root dry biomass over control was highest in *Trichoderma* treated seedlings through seed treatment (56.25 and 77.78 % respectively) followed by *Trichoderma* applied combining through seed treatment and soil application (43.75 and 56.56% respectively). Higher the root shoot ratio reveal better increase in root surface area and better establishment of crop in soil. The present result indicated that better root biomass in comparison to shoot was produced by the seedlings treated with combined inoculation of *Trichoderma* and *P. fluorescence* through seed treatment and soil application (R:S-0.220) which was followed by *Trichoderma* applied through soil application (0.217) and *Trichoderma* applied through seed treatment (0.213). Enhancement of shoot dry weight from 16-48% and root dry weight from 82-137% when inoculated with Fluorescent Pseudomonads [22].

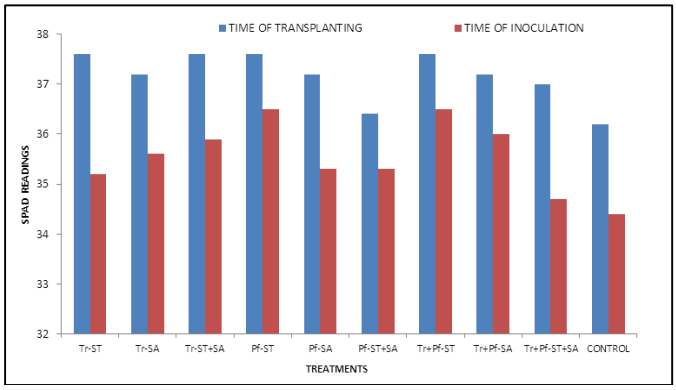

Fig 2: Variation in total chlorophyll in cabbage under influence of bio-control agents at 20 DAS and 45 DAS.

Variation in total chlorophyll in cabbage under influence of bio-control agents

Results on total chlorophyll as expressed in SPAD -502 readings at transplanting stage at 20 days after sowing and at pathogen inoculation time at 45 days after sowing, have been elucidated in fig.2 showed no significant variation within the bio-agent treated seedlings irrespective of delivery system and the total chlorophyll was ranged between (36.4-37.6) at transplanting state. Although these were significantly higher in comparison to control (36.2). Comparatively higher variation was found at the time of inoculation among the bio-treated plants had significantly higher total chlorophyll was measured in the plants developed with *P. fluorescence* applied through seed treatment and combined inoculation of *Trichoderma* and *P. fluorescence* applied through seed treatment. The rate of chlorophyll degradation was less in those two treatments. Aging of plants increases oxidative stress in chloroplasts and results in reduction of total chlorophyll in leaves [3].

Table 1: Effect of bio- inoculation on dry bio mass of cabbage seedlings

Treatment	Shoot dry wt. (mg/seedling)	Root dry wt. (mg/seedling)	Root: shoot	% increase in shoot dry wt. over control	% increase in root dry wt. over control
Tr-ST	0.075^{A}	0.016^{A}	0.213	56.25	77.78
Tr-SA	0.060^{BC}	0.013^{BC}	0.217	25.00	44.44
Tr-ST+SA	0.069^{AB}	0.014^{AB}	0.203	43.75	55.56
Pf-ST	0.067^{AB}	0.014^{B}	0.209	39.58	55.56
Pf-SA	0.061^{B}	0.011^{CD}	0.180	27.08	22.22
Pf-ST+SA	0.065^{AB}	0.013^{BC}	0.200	35.42	44.44
Tr+Pf-ST	0.066^{AB}	0.014^{B}	0.212	37.50	55.56
Tr+Pf-SA	0.063^{AB}	0.013^{BC}	0.206	31.25	44.44
Tr+Pf-ST+SA	0.059^{BC}	0.013^{BC}	0.220	22.92	44.44
Control	0.048^{C}	0.009^{D}	0.188		
SEm± CD(P=0.05)	0.00428 0.01219	0.00077 0.0021931			

*Alphabets in superscript represent DMRT

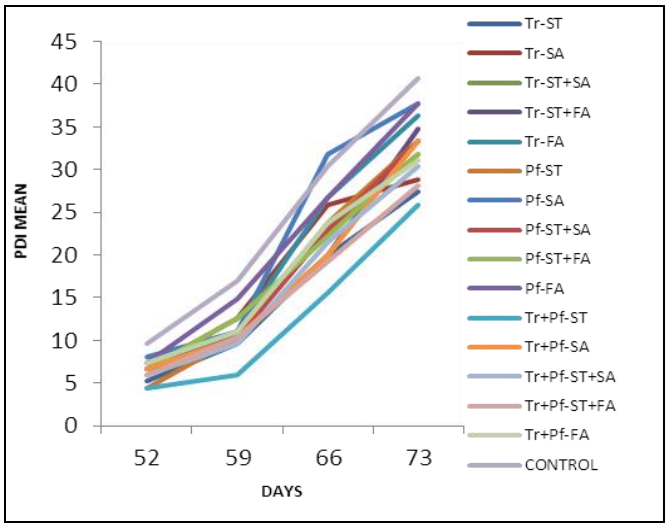

Fig 3: Effect of bio-inoculation on disease dynamics of Alternaria leaf spot in cabbage under challenge inoculation

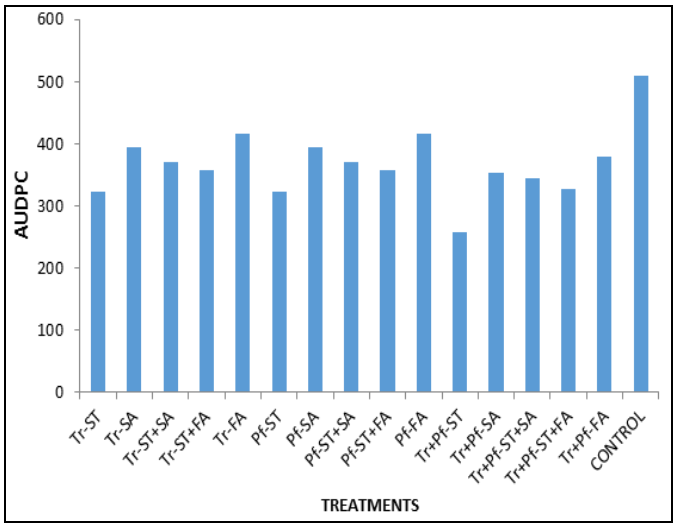

Fig 4: AUDPC of Alternaria leaf spot in bio-inoculated cabbage plants.

Effect of bio-inoculation on disease dynamics of Alternaria leaf spot in cabbage under challenge inoculation

The bio-inoculated plants inoculated with spore suspension of pathogen (*Alternaria sp*) at 45 days of crop age. Then after the severity was recorded up to 75 days of crop age at 7 days interval. The result have been presented in the table 2 and the trend has been depicted in fig.3. The results indicated that there was no significant difference in disease severity although the control showed comparatively higher disease severity 9.63%, whereas the bio-inoculated plants had the disease severity raged between (4.45-8.14%). Significant variation in disease development was found then after and it continued up to terminal stage of disease record 14 days after challenge inoculation lowest disease severity was recorded in the plants raised by combined inoculation of *Trichoderma* and *P. fluorescence* applied through seed treatment (5.93%) although it was statistically at par with the severity recorded in plants treated with *Trichoderma* as seed treatment and combined inoculation of *Trichoderma* and *P. fluorescence* through seed treatment along with soil application (9.63%). At this stage control plants showed 17.04 % and the extend of reduction in disease severity was ranged between 13.03 to 43.49% in bio-inoculated plants at 21 days after challenge inoculation. Lowest severity was recorded in plants raised by co inoculation of *Trichoderma* and *P. fluorescence* through seed treatment (15.56%) although it was statistically at par with the records from plants raised by *Trichoderma* through seed treatment and also of the plants raised through co-inoculation of *Trichoderma* and *P. fluorescence* through soil application. Interestingly the plants treated with *P. fluorescence* through soil application showed highest disease severity (31.85%) although it was statistically at par with control (30.37%). Soil application did not show any significant effect in reduction of disease severity at the terminal stage disease record i.e. 28 days of challenge inoculation. Lowest severity was recorded when both *Trichoderma* and *P. fluorescence* were applied through seed treatment and foliar application (28.15) and it was statistically at par with many other bio-inoculation treatments particularly when the biocontrol agents were applied through seed treatment. In control plants 40.74% disease severity was recorded which indicated that as high as 30.90% reduction in disease severity could be achieved through biocontrol agent application.

AUDPC for all the treatments was also calculated and significantly low AUDPC (Fig.4) was recorded where *Trichoderma* and *P. fluorescence* applied combined through seed treatment (256.67). This result was statistically at par with *Trichoderma* applied through seed treatment *Trichoderma* and *P. fluorescence* combined application through seed treatment and foliar application and *Trichoderma* and *P. fluorescence* through seed treatment and soil application (321.48, 326.67, 344.81, respectively). In control plants the calculated AUDPC was significantly higher (508.15) yet in few instances of biocontrol plants like, plants raised with *P. fluorescence* applied through soil application, *P. fluorescence* applied through foliar application. *Trichoderma* and *P. fluorescence* are well known for their biocontrol potential against several soil borne fungal pathogens [2, 7, 17] but in many instances they can so be active against many air borne fungal pathogens *Botrytis cinera*, *Alternaria sp* [12] and *Phytophthora infestance* [19] perhaps by elicitation of resistance and associated defense response of *Trichoderma sp* induce resistance against different plant pathogens through triggering systemic acquired resistance [10]

Table 2: Percent disease index (PDI) and Area under Disease Progress Curve (AUDPC) of Alternaria leaf spot in cabbage in bio-inoculated plants under challenge inoculation

Treatment	PDI in time scale after challenge inoculation				AUDPC
	7 days	14 days	21 days	28 days	
Tr-ST	5.19^{A}	9.63^{CD}	20^{CDE}	27.41^{BCD}	321.48^{5EF}
Tr-SA	6.67^{A}	12.60^{ABC}	25.92^{ABCD}	28.89^{BCD}	394.14^{BCDE}
Tr-ST+SA	5.93^{A}	11.11^{BCD}	22.97^{CD}	31.85^{ABCD}	370.74^{CDE}
Tr-ST+FA	6.67^{A}	10.37^{ABC}	20^{CDE}	34.81^{ABCD}	357.78^{CDE}
Tr-FA	6.67^{A}	11.11^{BCD}	26.67^{ABC}	36.33^{ABC}	414.81^{BCD}
Pf-ST	$4.45`^{A}$	10.37^{BCD}	23.70^{BCD}	33.33^{ABCD}	321.51^{CDE}
Pf-SA	8.14^{A}	11.11^{ABC}	31.85^{A}	37.78^{8AB}	394.14^{AB}
Pf-ST+SA	6.67^{A}	10.37^{BCD}	22.97^{CD}	33.33^{ABCD}	370.74^{CDE}
Pf-ST+FA	6.67^{A}	12.61^{ABC}	22.22^{BCD}	31.85^{ABCD}	357.78^{BCDE}
Pf-FA	7.41^{A}	14.82^{AB}	26.67^{ABC}	37.78^{AB}	414.81^{ABC}
Tr+Pf-ST	4.45^{A}	5.93^{D}	15.56^{E}	25.93^{CD}	256.67^{F}
Tr+Pf-SA	6.67^{A}	10.37^{BCD}	20^{CDE}	33.33^{ABCD}	352.63^{DE}
Tr+Pf-ST+SA	5.92^{A}	9.63^{CD}	21.48^{CDE}	30.37^{ABCD}	344.81^{DEF}
Tr+Pf-ST+FA	5.92^{A}	10.37^{BCD}	19.26^{DE}	28.15^{D}	326.67^{EF}
Tr+Pf-FA	7.40^{A}	11.11^{BCD}	23.71^{BCD}	31.11^{ABCD}	378.52^{CDE}
CONTROL	9.63^{A}	17.04^{A}	30.37^{AB}	40.74^{A}	508.15^{A}
SEm±	1.9685	1.6667	2.5660	3.7406	29.709
CD(P=0.05)	NS	4.81	7.41	10.80	85.78

*Alphabets in superscript represent DMR

Conclusion

Bio-inoculated seedlings exhibited significantly higher vigour index. Single or combination of *T. harzianum* and *P. fluorescence* applied through seed treatment resulted comparatively higher seedling vigour. Seed treatment along with soil application did not provide better result as compared to seed treatment alone.

Higher shoot biomass was observed when *Trichoderma* was applied through seed treatment. Root dry biomass also followed the same trend. Increase in shoot and root dry biomass over control was highest in *Trichoderma* treated seedlings applied through seed treatment.

Higher total chlorophyll was measured in the plants developed with *P. fluorescence* applied through seed treatment and combined inoculation of *Trichoderma* and *P. fluorescence* applied through seed treatment. The rate of chlorophyll degradation was less in those two treatments.

Significant variation in disease development was found then after and it continued up to terminal stage of disease record. Fourteen days after challenge inoculation, lowest disease severity was recorded in the plants raised by combined inoculation of *Trichoderma* and *P. fluorescence* applied through seed treatment. Soil application did not show any significant effect in reduction of disease severity at the terminal stage disease record i.e. 28 days of challenge inoculation. Lowest severity was recorded when both *Trichoderma* and *P. fluorescence* were applied through seed treatment and foliar application. In control plants 40.74% disease severity was recorded which indicated that as high as 30.90% reduction in disease severity could be achieved through biocontrol agent application. Low AUDPC was recorded were *Trichoderma* and *P. fluorescence* applied combined through seed treatment. In control plants the calculated AUDPC was significantly higher.

Before challenge inoculation the highest protein concentration was measured in plants raised with combined inoculation of *Trichoderma* and *Pseudomonas fluorescence* applied through seed treatment and foliar application (5.49 mg/g of fresh wt.). After challenge inoculation highest increase was observed in the plants raised with *P. fluorescence* applied through seed treatment and soil application (116.72%) followed by *Trichoderma* applied through foliar application (107.17%). In general *P. fluorescence* was found to be more potential protein inducer compared to *Trichoderma.*

References

1. Abdul-Baki A, Anderson JD. Vigour determination in Soybean seed by multiple criteria. Crop Science, 1973; 13: 630-633.
2. Barbosa MA, Rehn KJ, Menejeds M and Mariano RL. Antagonism of *Trichoderma sp* on *Cladosporium harbabarium* and their enzymatic characterization. Brazelian Journal of microbiology, 2001; 32:98-104.
3. Bosh N and Alegre L. Plant aging increases oxidative stress in chloroplasts. Planta. 2002; 214(4): 608-615.
4. Chaddha KC and Brown SA. Biosynthesis of phenolic acids in tomato plants infected with *Agrobacterium tumefaciens.* Canadian J. Bot. 2001; 52:2041–2047.
5. Conn KL, Tewari JP. Survey of Alternariablackspot and Sclerotinia stem rot in central Albertain. Can. Plant Dis. Survey, 1990; 70:66-67.
6. Dharmendra K, Maurya N, Bharati YK, Kumar A, Kumar K, Srivastava K, Chand G, Kushwaha C, Singh SK, Mishra RK and Kumar A. Alternaria blight of oilseed Brassicas: A comprehensive review. African Journal Microbiology Res. 2014*;* 8(30):2816-2829.
7. Dolatabadi KH, Goltapeh EM, Varma A, Rohani N. *In vitro* evaluation of arbuscularmycorrhizal-like fungi and Trichoderma species against soil borne pathogen. Journal of Agricultural Technology 2011; 7(1):73-84.
8. FAO. 2013. The state of food in security in the world 2013, 6.
9. Hamilton RH. A corn mutant deficient in 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one with an altered tolerance of atrazine. Weeds, 1964; 12: 27-30.
10. Harman GE, Howell CR, Viterbo A, Chet I and Lorito M. Trichoderma species–opportunistic, avirulent plant symbionts. National Review of Microbiology, 2004; 2:43–56, 10.
11. Humperson-Jones FM, Phelps K. Climatic factors influencing spore production in *Alternaria brassicae* and *Alternaria brassicicola.* Ann. Applied Biology, 1989; 114:449-458.
12. Kucuk C, kivanc M. Isolation of Trichodermaspp and determination their antifungal biochemical, physiological features.Turkish Journal of Biology, 2003; 27:247-253.
13. Mac KSL, Keifer P, Ayer WA. Components from the phytotoxic extracts of *Alternaria brassicicola,* a black spot pathogen of canola. Phytochemistry, 1999; 51:215-221.
14. Meah MB, Hau B, Siddique MK. Relationship between disease parameters of alternaria blight (*Alternaria brassicae*) and yield of mustard. Journal of Plant Diseases and Plant Protection, 2002; 3:243-251.
15. Rangel JF. Two Alternaria disease of cruciferous plants. Phytopathology 1945; 35:1002-1007.
16. Sharma P, Jha AB, Dubey RS and Pessarakli M. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of Botany, 2004, 1-26.
17. Siddiqui IA and Shaukat SS. Rhizobacteria-mediated induction of systemic resistance (ISR) in tomato against *Meloidogyne javanica.* J. Phytopathology, 2002; 150: 469-473.
18. Singh T, Saikia R, Jana T, Arora DK. Hydrophobicity and surface electrostatic charge of conidia of the mycoparasitic Trichoderma species. Mycological Progress, 2001; 3(3):219–228.
19. Tra H, Ficke A, Asiimwe T, Hofte M, Araaijmaker JM. Role of cyclic lipopeptide Massetolide A in biological control of *Phytophthora infestans* and in colonization of tomato plants by Pseudomonas fluorescence. New Phytologist 2007; 175:731-772.
20. Valkonen JPT, Koponen H. The seed-borne fungi of Chinese cabbage (*Brassica pekinensis*), their pathogenicity and control. Plant Pathology 1990; 39:510-516.
21. Verma PR, Saharan GS. Monograph on Alternaria diseases of crucifers. Agriculture Canada Research Station, Saskatoon, Technical Bulletin. No. 1994-6E, 162.
22. Walley FL, Germida JJ. Plant growth promoting rhizhobacteria alter rooting pattern and arbuscularmycorhizal fungi colonization of field grown spring wheat. Biology and fertility of soils, 1997; 23:113-120.
23. Wilcoxson RD, Skovmand B, Atif A. Evaluation of wheat cultivars for ability to retard development of stem rust. Annals of Applied Biology, 1975; 80: 275-281.