Int J Pharm Bio Sci 2014 April ; 5 (2) : (B) 680 - 688

Research Article **Bioinformatics**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

SYMPTOMOLOGICAL, CULTURAL AND MOLECULAR VARIABILITY OF *ALTERNARIA BRASSICICOLA* LEAF SPOT IN BROCCOLI (*BRASSICA OLERACEA* VAR. *ITALICA* L.)

GIREESH CHAND[1] AND K. K.CHANDRA*[2]

[1]*Department of Plant Pathology, Bihar Agricultural University, Sabour, Bhagalpur-813210, India*
[2]*Deparment of Forestry,Wildlife and nvironmental Sciences Guru Ghasidas Central University, Bilaspur – 495009 (Chhattisgarh), India*

ABSTRACT

Variation of *Alternaria brassicicola* leaf spot of Broccoli (*Brassica oleracea* var. *italica* L.) was evaluated based on morphological, cultural and molecular parameters during Rabi season 2010 and 2011. Six isolates of *Alternaria* were cultured in vitro using different growth medium and the growth pattern of the fungi was studied. Maximum 86 leaf spots were recorded from Naini followed by BHU (74) and minimum 17 spots from CSA samples. The size of spots shown variations in different isolates collected from different places. The leaf spots were followed the reducing trend with the increasing number of spots. The maximum 0.5-1.9mm size of spot was noticed in CSA samples and minimum 0.2-0.7mm from Naini sample. The colour of spots was light olivacious, brown with concentric rings with black pin point at the centre. The conidial length ranged 13-120 µm, width 6-16 µm with 2-8 septa. The growth of *A. brassicicola* recorded maximum in host extract media followed by Potato Dextrose Agar while the growth observed minimum in Czapek's medium. The variations also exhibited in protein profiling by using SDS-PAGE. The highest molecular weight of protein was detected in CSA (22kDa) isolates followed by isolates from ND (21kDa) and BHU (21kDa) while minimum 20kDa of protein was recorded in Naini isolate.

KEY WORDS: **Alternaria, Leaf blight, Molecular weight; Broccoli**

K. K.CHANDRA
Deparment of Forestry,Wildlife and Environmental Sciences
Guru Ghasidas Central University, Bilaspur – 495009 (Chhattisgarh), India

INTRODUCTION

Broccoli (*Brassica oleracea* var*. italic*) is one of the important vegetable in India ranked second in global production. It accounts 5MT production taking the place of cauliflower among medium to rich income families due to the presence of high nutrients and medicinal properties. Besides high nutritive values broccoli also contains some glucosinolet substances which activate some enzymes in our body that inhibits cancer causing agent. The productivity of this crop in India is about 260q ha^{-1} against the world average 275q ha^{-1}. In comparison to cauliflower the productivity of Broccoli is very less though both belongs to the family Brassicaceae distinguished only by the presence of multiple flower buds in broccoli rather than a single curd in cauliflower. The production of broccoli may be increased up to 33 to 35 percent by the inclusion of integrated nutrient and pest management practices. One of the major causes is the leaf spot diseases caused by *Alternaria brassicicola*. It is cosmopolitan in their distribution reported in all continents and identified as most damaging fungal disease (Ghose et al. 2008). The yield losses have been reported in the range of 32-69 percent by this fungus (Surviliene et al. 2004; Shrestha et al. 2005). The disease decreases the nutritive value of this vegetable, their storability and also decreases the resistance of vegetable to rot. The first appearance of the disease start on the leaves as necrotic lesions often describes as black and sooty with chlorotic yellow halos surrounding the lesions site in wet season. *A. brassicicola* is a necrotrophic plant pathogenic fungus secretes toxic secondary metabolites and proteins that cause cell death (Ravindra, 2013). In recent years the incidence of this disease reported very severe losses which pose a new threat to *Brassicaceous* vegetable cultivar. Due to fluctuating in environmental conditions the pathogen does not have a uniform growth rate. The pathogens greatly influenced by weather condition. Studies on pathogenic variability have the foundation for the development of pre-breeding populations as strategic defence mechanism (Vishwanath et al. 1999). Researches on *A. brassicicola* in Broccoli are still scarce, therefore field identification, bioformulations and disease forecasting module is not developed so far. The present study was undertaken to characterize the infection behavior, pathogenic growth pattern caused by *A. brassicicola* under different growing medium for better understanding of the fungus.

MATERIALS AND METHODS

Collection, Isolation, Purification and Maintenance of Alternaria brassicicola

The large number of dark blighted leaf samples of Broccoli were collected from different locations of NDUA&T (Narendra Deva University of Agriculture and Technology), Faizabad; BHU (Banaras Hindu University) and IIVR (Indian Institute of Vegetable Research), Varanasi; CSAU&T (Chandra Sekhar Azad University of Agriculture and Technology), Kanpur; SHIATS (Institute of Agriculture and Technology), Naini, Uttar Pradesh, India conducting field survey during Rabi 2010 to 2011 and coded ND, BHU, IIVR, CSA and Naini respectively. The spotted leaves showing disease symptoms were screened out for isolation of the pathogen. The pathogen was identified on the basis of its morphological and cultural characters with the help of key as per Chand et al. (2007) as well as the pathogenicity to the host were also assessed. The culture was purified using single spore technique and maintained at 2% Potato Dextrose Agar slant at 6-8 ^{o}Ċ.

Morphological variability of the pathogen

Morphological characteristics of five representative isolates of *Alternaria* spp. including shape, size of conidia and conidiophores was measured at 40X magnification using calibrated filler micrometer in the microscope. Number of transverse and longitudinal septa was also counted.

Cultural variability of the pathogen
The variations in growth pattern, color of colony were recorded separately for Potato Dextrose Agar (PDA), PDA+$CaCO_3$, Host Extract Agar, Czapek's Agar and Richard's Agar mediums. These isolates were incubated at 25±2°Ċ with 95±5% humidity and data on parameters were recorded on 4th and 7th day after inoculation.

Molecular variability
Alternaria brassicicola grown on potato dextrose broth at room temperature and mycelium was harvested after 7th day of inoculation and lyophilized. The fungal isolates were protein electrophorsed on 10% acrylamide gel as per method given by Laemmli (1970). Isolates were observed by Gel Electrophoresis SDS-PAGE method and observed the amount of protein found in five representative isolates on the basis of their molecular weight (Naik et al. 2010; Jadhav et al. 2011). Total proteins were extracted in 5ml phosphate buffer (0.5M with 7.0 pH). 2g dried mycelium was macerated in pestle mortar and crude sap was filtered through muslin cloth. The filtrate was centrifuged at 6000 rpm for 20 minutes and the supernatant was used in the study. Prior to loading the sample on gel, the dye was added and denatured by keeping the sample on a water bath for 6 minutes. It was cooled at room temperature and chilled before loading on gel. Vertical gel system (Genie) 8X7cm was used for gel electrophoresis. The gel plate and spacers were cleaned by ethanol, dried, assembled and finally the chamber was sealed using 2% water agar. A prepared gel mixture 10% was poured into the chamber between glass plates, leaving space from the top and allowed polymerization for 1 hour then 4% stacking gel mixture was filled in the remaining portion of the chamber and a comb was placed immediately in the stacking and allowed further polymerization for 1 hour period. After complete polymerization of stacking gel, the comb was removed and buffer tank was filled with electrode buffer (pH- 8.2) without distorting the shape of the well. The standard protein marker (Genie, 14.3- 97 kDa) and loading dye was mixed at 1:2 ratio. Marker and sample were loaded separately on electrophoresis unit and gel was run at 40V for 1 hour and then increased upto 80V. After complete run, gel was separated carefully from the gel plates and immersed in staining solution for five hours using continuous shaker and then transferred in a tray containing de-staining solution. De-staining solution was frequently changed until the background of the gel become colorless, the gel was photographed and documented with the help of densitometer.

RESULTS AND DISCUSSION

Symptomatological variability
Symptom parameters of the leaf spots exhibited variations in growth pattern, size and even in number of spots collected from different places under the study. Maximum 86 numbers of spots were recorded in the samples collected from SHIATS, Naini, Allahabad followed by the samples of BHU (74) and minimum 17 spots were recorded in the samples from CSA (Fig.1). The size of spots ranged 0.2-1.9mm with light olivaceous, brown colour along with concentric rings with black pin point at centre. The size of spots observed maximum at CSA and minimum at SHIATS. The size of the spots observed larger with decreasing the number of spots (Table 1). Ellis (1973) reported the spot size of this fungi upto 1.8mm with light olivaceous, brown colour and profuse sooty spores. Number of disease spot and their size was observed variation among different study sites as also confirmed by Chand et al. (2007) due to variations in temperature, humidity and other environmental factors.

Morphological variability
Morphological study of all isolates exhibited well-formed conidiophores of the fungus either singly or in groups, straight or flexuous and brown to olivaceous colour. The conidia were muriform, olivaceous brown colour with nonexistent beaks. The size of conidia recorded 13-120µm in length and 6-16µm in width. The highest 4-9 conidial chain was recorded in CSA isolates and lowest 2-3 chains in isolated from ND (Table 2). The shape of the conidia was cylindrical to obclavate with cross and

longitudinal septa. The cross septa was examined maximum 2-8 in SHIATS isolates and minimum 2-3 in IIVR isolates (Fig. 2). Joly (1959) also propounded similar results and prepared a synaptic key for identification of *Alternaria*. Moreover Mehrotra and Narain (1969) also reported *Alternaria* species on various hosts with 3-4 chained with nonexistent beak. Similar variations in different isolates of *Alternaria* might be due to the genetic variability of the fungi as also reported by Mora and Earle (2001).

Cultural variability

The isolates of *A. brassicicola* also differed with respect to colour and growth pattern. Isolates of CSA recorded maximum growth on host extract agar followed by PDA+CaCO3. Tong Yunhui et al. (1994) observed variations in growth and pattern of colony development of *Alternaria* under different media's in vitro condition. The temperature between 25-30°C was found optimum for the growth and sporulation of *Alternaria* as also reported by Kaul and Saxena (1988) in case of *Alternaria solani*. The maximum 8-15mm radial growth was found in fungus on Host Extract Agar while minimum 7-8mm on Richard's medium in 4 days after inoculation (Graph 1). Similarly in PDA the radial growth was maximum 15mm in CSA isolates (Table 3) while remaining isolates exhibited similar radial growth (8mm). The colony colour recorded white in PDA and Host Extract Agar and gray brown colour in Czapek's media. Exceptionally the SIAHTS and CSA isolates showed light black and grey white colours respectively in Host Extract Agar media. The variation in radial growth and colour of the fungus colony was observed more significant after 7 days of inoculation (Table 4). During this period the fungal growth ranged 15-26 mm in Host Extract Agar media and 14-15mm in PDA with black colonies. In Richard's medium and Czapek's Agar medium the growth and colour ranged 14-16mm with dark brown colony with all isolates except SHIATS which shown gray brown colour on Czapek's Media. In PDA+ CaCO3 Media the growth recorded maximum of 18mm from ND isolates and minimum of 15mm from BHU isolates. The colour of fungal colonies were noticed brown, light brown and dark brown in IIVR, CSA and SHIATS isolates respectively. The fungal growth and colour was greatly influenced by the site temperature, humidity and soil moisture during the growing time of Broccoli. Therefore, the variation may exist in color and growth pattern of the fungus in different places. In India *Brassicas* are sown from late August to November and harvest from February , March depending on the area, coupled with harboring of the fungal pathogen by other *Brassica* vegetables could be the reason for carryover of the *A. brassicae* from one crop to another and air borne spores of *A. brassicae* from the primary source of inoculums of this polycyclic disease exhibited variation in growth pattern and colour. Variation among the Indian isolates of *Alternaria* was also noted by Meena et al. 2005).

Molecular variability

The highest molecular weight of the fungus was recorded (22 kDa) in the samples collected from CSA followed by NDUAT (Fig. 3) and BHU (21 kDa) indicated very high virulency of the fungus to break the resistance of the host and higher yield loss in broccoli. This was in confirmation of earlier report by Chand and Chakrabarti (2003). The minimum molecular weight of the protein was found in SHIATS sample might be due to the higher resistance of the host as well as the poor virulency of the fungus in this area. Similarly Mora and Earle (2001) also observed the high kDa due to higher endochitinase activity in transgenic Brassica varieties than control varieties. The variations in different isolates of *Alternaria* in various sample areas were might be due many factors such as the genetic variations of the fungus, variation in environment and also due to the different varieties of broccoli grown in sampled areas. It was concluded from the present study that different isolates of *Alternaria* may exhibits differential growth pattern and symptom in different areas even in same hosts. The fungus growth was highest and fastest in CSA sample and minimum in SHIATS, Naini, sample. Similarly the colour, conidial structure and other

morphological parameters of the leaf blight *Alternaria* of Broccoli showed variability. The variation was also found in molecular level of protein in which CSA isolates expressed highest kDa and virulence to host as compared to other isolates of the study.

Table - 1
Number, size and colour of the leaf spot samples in Broccoli.

Isolates	No. of spot	Colour of spot	Spot size in diameter (mm)	Yellow hollow
ND	48± 5.30	Light brown	0.3 - 1.3	Absent
CSA	17± 3.50	Olivaceous brown	0.5 – 1.9	Absent
IIVR	30± 5.22	Dark brown	0.4 – 1.8	Present
BHU	74± 4.60	Olivaceous brown	0.2 – 1.8	Present
NAINI	86± 4.05	Dark brown	0.2 – 0.7	Present

Table- 2
Morphological variations in different isolates of Alternaria brassicicola.

Morphological Characteristics	SAMPLE ISOLATES				
	ND	CSA	IIVR	BHU	NAINI
Conidiophore Colour	olivaceous brown	pale olivaceous brown	olive brown	olive brown	pale olivaceous brown
Conidiophore Shape	simple erect and curved septate	simple erect	curved, septate	simple erect	Simpleer ect
Length (µm)	24-76	25-80	25-75	25-75	24-75
Width (µm)	4-8	5-10	3-8	4-8	5-10
Conidia					
Conidia in chain	2-3	4-6	4-8	5-8	4-6
Shape	Cylindrical to obclavate	Cylindrical to obclavate	Cylindrical	Cylindrical to obclavate	Cylindrical to obclavate
Cross septa	2-5	2-6	2-8	4-6	2-7
Longitudinal septa	1-2	1-3	1-2	1-2	1-2
Length (µm)	15-120	20-120	20-110	13-108	15-110
Width (µm)	6-13	6-15	8-16	6-15	6-16
Beak	Non-existent	Non-existent	Non-existent	Non-existent	Non-existent

Table 3
The growth and colour of Alternaria brassicicola under different growing medias after 4 days.

Isolates	Host extract		PDA		PDA+CaCO3		Richard' medium		Czapek's Medium	
	Growth (mm)	Colour	Growth (mm)	Colour	Growth (mm)	Colour	Growth (mm)	Colour	Growth (mm)	Colour
ND	18 (2.11)	White	24 (3.33)	Dull white	10 (1.22)	Greenish black	7 (0.25)	Light brown	6 (0.15	Gray brown
CSA	23 (3.50)	White	29 (1.55)	White	11 (0.88)	Light brown	8 (1.11	Gray black	11 (0.55)	Gray white
IIVR	24 (2.21)	White	20 (3.50	Dull white	18 (1.76)	Pale brown	6 (0.55)	Light brown	8 (1.03)	Gray brown
BHU	14 (3.00)	White	20 (2.44)	Dull white	8 (0.50)	Brown	8 (1.20)	Black	7 (1.00)	Gray brown
NAINI	25 (3.68)	Pale black	25 (3.10)	Dull white	10 (1.00)	Light black	7 (0.87)	Light brown	10 (0.88)	Gray brown

Table 4
Effect of different media on radial growth and colour of Alternaria brassicicola after 7 days.

Isolates	Host extract		PDA		PDA+CaCO3		Richard' medium		Czapek's Medium	
	Growth (mm)	Colour	Growth (mm)	Colour	Growth (mm)	Colour	Growth (mm)	Colour	Growth (mm)	Colour
ND	36 (3.57)	Black	38 (2.30)	Black	36 (2.20)	Gray white	14 (0.55)	Dark brown	12 (1.30)	Dark brown
CSA	40 (2.80)	Black	40 (3.50)	Black	34 (3.18)	Light brown	20 (2.03)	Dark brown	14 (0.55)	Dark brown
IIVR	38 (4.00)	Black	36 (2.60)	Light brown	30 (2.55)	Brown	25 (0.77)	Dark brown	11 (0.88)	Dark brown
BHU	39 (3.05)	Black	37 (3.12)	Black	38 (1.50)	Gray white	13 (1.00)	Black	13 (0.55)	Dark brown
NAINI	37 (4.40)	Black	38 (2.50)	Black	35 (1.80)	Dark brown	16 (0.56)	Dark brown	15 (1.20)	Gray brown

Graph 1
Mean radial growth of A. brassicicola in different mediums isolated from Broccoli.

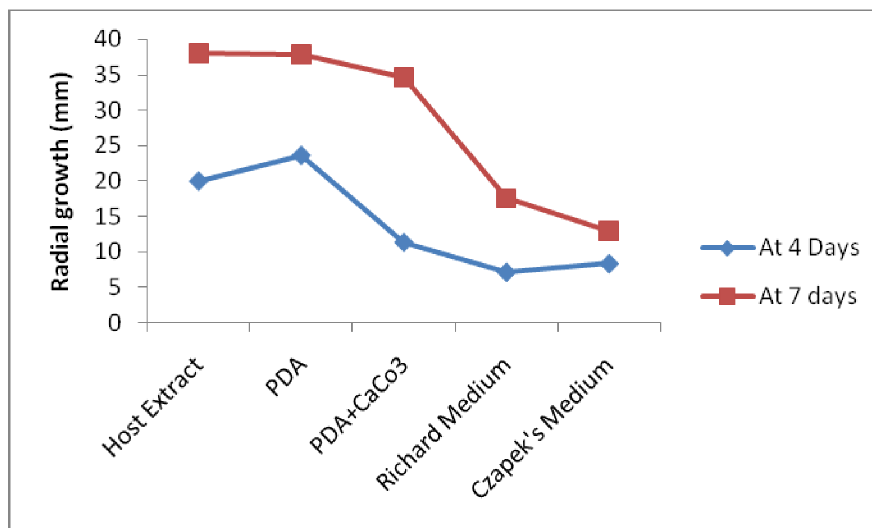

Figure 1
Spotted leaf sample of Broccoli collected from selected places.

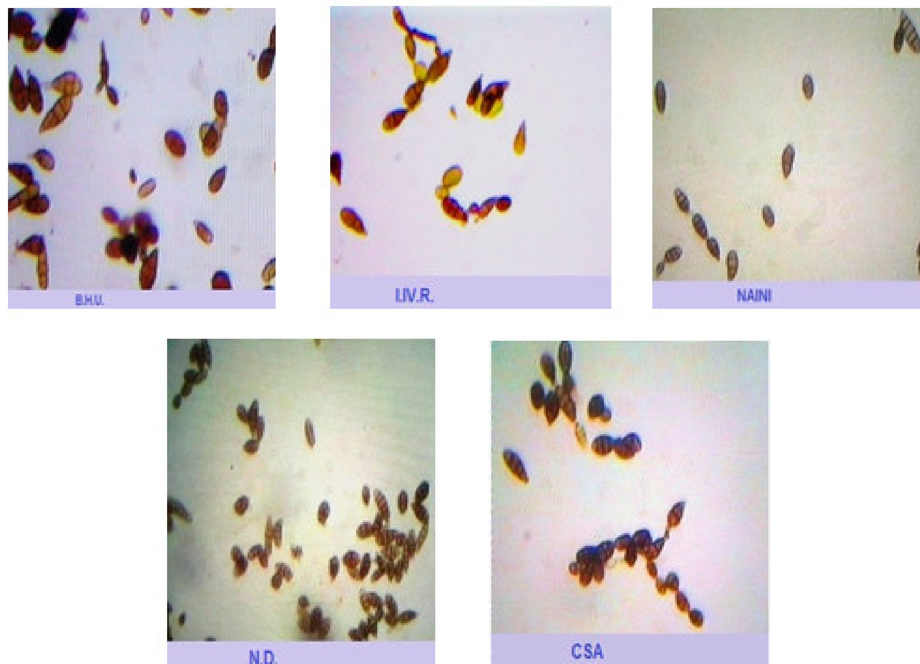

Figure 2
Structure of Conidia of A.brassicicola isolates of different places.

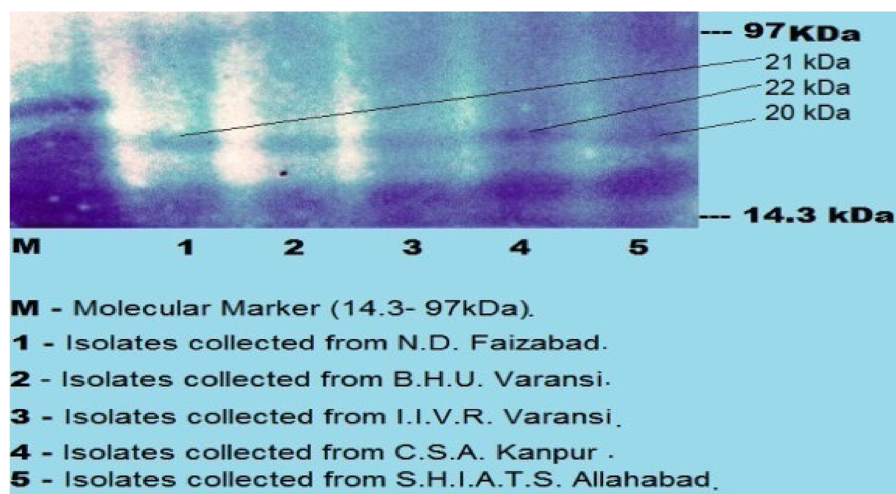

Figure 3
PCR amplifications of A. brassicicola in broccoli.

REFERENCES

1. Chand, G, Narain, U, Kumar, M and Verma, S. 2007. Symptomatology, Etiology and Ecofriendly Management of Alternaria leaf spot of Broccoli. In: S. Ahmad and U. Narain (eds.) *Ecofriendly Management of Plant Diseases*, Daya Publishing House New Delhi, pp.: 461-472.
2. Ghose, A, Dey, S, Barton, H, Loake, GJ and Basu D. 2008. Differential profiling of selected defence related genes induced on challenge with *Alternaria brassicicola* in resistant while mustard and their comparative expression pattern in susceptible Indian mustard. *Mol Pl Pathogen,* 65: 763-775.
3. Ellis, MB. 1973. *Dematiaceous Hyphomycetes*. Commonwealth Mycological Institute, Kew, Surrey, England, pp. 464-497.
4. Jadhav, BM, Perane, R and Pawar, NB. 2011. Morphological, pathological and molecular variability among *Alternaria macrospora* isolates causing leaf blight of cotton. *Indian Phytopath,* 64(3): 254-257.
5. Joly, P. 1959. Morphological variation and the ideas of species in the genus *Alternaria. Bulle Soc Mycol Fr.*, 75: 149-158.
6. Kaul, AK and Saxena, HK. 1989. Conidial morphology of isolates of *Alternaria solani* showing cultural and pathogenic variability. *Pl Dis Res.,* 4: 184-186.
7. Laemmli, EK. 1970. Cleavage of structure protein during the assembly of the head of bacteriophage T_4 .. *Nature*, 227: 680-685.
8. Mehrotra, BS and Narain, U. 1969. Studies on the genus *Alternaria*: Some new record and a new species. *Indian Phytopath Soc Bulle*. 5: 1-7.
9. Meena, PD, Chattopadhyay, C, Kumar, VR, Meena, RL and Rana, US. 2005. Spore behaviour in atomosphere and trends in variability of *Alternaria brassicae* population in India.Global conference II on plant health- global Wealth organized at MPUAT, Udaipur, 25-29 Nov 2005, p. 91.
10. Mora, AA, Earle and ED. 2001. Resistance to *Alternaria brassicicola* in transgenic broccoli expressing a *Trichoderma harzianum* endochitinase gene. *Mole Breed*., 8: 1-9.
11. Naik, MK, Prasad, Y, Bhat, KV and Devikarani, GS. 2010. Marphological, Physiological, Pathogenic and molecular variability among isolates of *A. solani*. *Indian Phytopath.*, 63(2): 168-173.
12. Shrestha, SK, Munk, L and Mathur, SB. 2005. Role of weather on *Alternaria* leaf blight disease and its effect on yield and

yield component of mustard. *Nepal Agric Res J.*, 6:62-72.

13. Surviliene, E, Sidlauskiene, A, Vasinauskiene, M, and Zitikaite, I. 2004. Resistance of *Brassica* L. vegetable to phytopathogens. *Horti and Vegetable growing*, 23(1): 115-125.
14. Tong, Y, Liang, J, Tong, YH and Liang, JN. 1994. Study on the biology and pathogenicity of *Alternaria solani* on tomato. *J Jaingsu Agric College,*15: 29-31.
15. Vishwanath, Kolte, SJ, Singh, MP and Awasthi, RP. 1999. Induction of resistance in mustard (*Brassica juncea*) against *Alternaria* black spot with an avirulent *Alternaria brassicae* isolate - D. *Euro J Pl Pathol.,* 105:217-220.
16. Yadav, VK. 2012. Studies on variability of *Alternaria* leaf spot of Broccoli. In: M. Sc. Thesis, N. D. University of Agriculture and Technology, Kumarganj, Faizabad, (Uttar Pradesh) India. p. 132-147.
17. Jach, G, Gornhardt, B, Mundy, J, Legemann, J, Pinsdorf, E, Leah, R, Scheli, J and Mass C. 1995. Enhanced quantitative resistance against fungal disease by combinational expression of different barley antifungal protains in transgenic tobacco. *Plant Journal*. 8: 97-109.
18. Ravindra, K.N., Narayanappa, M. and Sharanappa, P. 2013. Study of endophytic fungal community from bark of Ventilago madrasapatna Gaertn. International Journal of Pharma and Bio Sciences 2013 Jan; 4(1): (B) 309 – 316.