*Invitro*evaluation ofefficacyof*Trichodermaharzianum* ontheradialgrowth of *Alternariaalternata*

ABSTRACT

*Stevia rebaudiana*Bertoni, a herbaceous perennial known for its natural sweetness, has garnered global recognition and is found in various regions of India. A study was carried out to investigate the leaf spot disease in Stevia, caused by *Alternariaalternata*(FR.) Keissler to find out the most suitable design of dual culture technique. Since the leaves are the primary site for synthesizing sweet glycosides, in Stevia this disease leads to significant losses and ultimately reduces the yield which leads to a serious concern. Due to the harmful effects of chemicalfungicides,findingasaferalternativetocontrolthepathogenbecameapriority.This prompted experiments with bioagents for pathogen control. Bio fungicides derived from *Trichoderma* are increasingly being recognized as successful agricultural applications, with over 50 registered products available worldwide. The present study was conducted in the Laboratory, Department of Plant Pathology, Naini Agricultural Institute, Sam Higginbottom University of Agriculture, Technology and Sciences, Prayagraj (Uttar Pradesh). The dual culture technique was carried out on Completely Randomized Design (CRD) with three replicationsandsixtreatments.Examinationoffungalcolonycharacteristicswasdonethrough microscopicexamination.At7DAI,maximummycelialinhibitionof96.70%wasrecordedin thetreatmentT_6(sixdiscsof*Trichodermaharzianum*againstonediscof*Alternariaalternata*).

Keywords:*Alternariaalternata*,bioagent,dualculture,fungalcolony,Stevia,*Trichoderma harzanium*,

1.INTRODUCTION

Stevia (*Stevia rebaudiana*Bertoni) is a member of the Asteraceae family and is recognized as one of the most significant natural sweetener plants with low-calorie content. India is known as the “Diabetes Capital” for its increasing number of diabetes cases. Stevia emerged as the realm of sweetness to all the diabetic individuals. The sweetness of stevia is due steveoside and rebauside. Maximum steveoside content is present in the leaves of stevia, emerging it as the main economic part. The global stevia market expanded significantly, increasingfrom₹ 61.65billionin2022to₹ 67.40billionin2023,reflectingacompoundannual growth rate of 9.9%. The increasing demand for natural sweeteners has encouraged Indian farmerstocultivatesteviaonalargescale**(Dhange*etal.*,2023)**.Currently,thenationproduces roughly 900 tonnes of dry leaf annually. A major concern is leaf spot disease caused by the infestation of *Alternariaalternata*(FR.) Keissler. Since the leaves are the primary site for synthesizing sweet glycosides, this disease leads to significant losses and ultimately reduces theyield**(Nayak*etal.*,2023)**.Thenewsurveyconductedoverthepastfiveyearsindicatesthat Alternaria leaf disease has been prevalent in medicinal plants cultivated invarious districts of West Bengal, India **(Chirutala and Simon, 2021)**. Alternaria diseases primarily impact the leaves,stems,flowers,andfruitsofannualplants,particularlyvegetablesandornamentals.The pathogenicfungus*Alternariaalternata*canproduceendo-polygalacturonase(endo-PG)and pectatelyase (PL) enzymes. These enzymes are responsible for breaking down pectic componentsoftheplant cellwall.Dependingon theinteractionbetweentheplantspeciesand the microorganism (compatible or incompatible), these pectinases may function as part of the fungal infection mechanism ortriggerahypersensitiveresponsebyreleasingoligosaccharides

thatactaselicitorsoftheplantresponse**(Roco*etal.*,2001)**.Biologicalcontrol,whichinvolves using living organisms (antagonists) to reduce pathogen activities, is a highly promising approachformanagingplantdiseases.Unlikechemicalfungicides,biologicalcontroldoesnot lead to the development of resistance in pathogens, nor does it contaminate the environment. Additionally, it meets the requirements of profitable markets. Among 25 fungal antagonists studied, the genus *Trichoderma* was identified as having the greatest biocontrol potential **(Ferreira and Musumeci, 2021)**. *Trichoderma* is a prominent and widely distributed filamentous fungus found in soil, where it plays a role in decomposing vegetative materials, plantmatter,andwood.*Trichoderma*isconsideredanexceptionalbiocontrolagentbecauseof its distinctive traits, including its rapid multiplication, widedistribution,andeaseofisolation andcultivation.Thecontrolmechanismsexhibitedby*Trichoderma*spp.varydependingonthe fungal species and environmental conditions. *Trichoderma* spp. utilize direct biocontrol mechanisms, including mycoparasitism, competition for space and nutrients, production of antimicrobial compounds (antibiosis) and lytic enzymes. They also employ indirect mechanisms, such as induction of systemic resistance, growth promotion, and rhizosphere competence.Variousstudieshavebeenconductedboth*invitro*and*invivo*using*Trichoderma harzianum* against *Alternariaalternata*. This study is conducted to know the most suitable designindualculturetechniqueusing*Trichodermaharzianum*against*Alternariaalternata*for maximum mycelial inhibition of the pathogen.

2.1 MATERIALANDMETHODS

2.1.1 ExperimentalSite

TheexperimentwasconductedintheLaboratory,DepartmentofPlantPathologyatNaini AgriculturalInstitute,SamHigginbottomUniversityofAgriculture,TechnologyandSciences, Prayagraj (Uttar Pradesh). The dual culture technique was carried out on Completely Randomized Design (CRD) with three replications and six treatments.

2.1.2 Isolation of Pathogen

For isolating and culturing of pathogen, Potato Dextrose Agar (PDA) medium was used. Diseased portion of the leaf was cut under aseptic conditions into small bits into a sterile dish withtheaidofscissorswhichwasflamedover aspiritlampflameandsurfacesterilizedin0.1 % sodium hypochlorite. The cut diseased and surface sterilized bits with 70% ethanol was placedonPetridishespouredwithsolidifiedpotatodextroseagar(PDA).Theinoculatedplates were incubated at room temperature until visible growths are seen on the plates. The fungal coloniesgrowingintheincubatedplateswassub-culturedintofreshmediumuntilpurecultures are obtained **(Rahman *et al.*, 2020)**. Sub-culturing was done at regular intervals, by using single spore method purification of the culture was made **(Toussoun and Nelson, 1976)**. The pure culture was maintained in slants and stored at 4°C temperature in the refrigerator.

2.1.2 Identification

Examinationofthefungalcolonycharacteristicswasdonethroughmicroscopicexamination. Using a sterile needle, a small portion of the culture was taken and placed on a sterile glass slide. It was stained using lactophenol and cotton blue and were identified using the key of **(Keissler, 1912; Barnett and Hunter, 1973)**~~**Barnet and Hunter**~~.

2.1.3 MorphologicalCharacteristics

Conidiophores were simple, light brown, variable in length ranging from 17.10 to 61.56 µm and mostly 2-3 septate rarely 4-5 septate. Conidia were found light to dark brown in colour,uniformwith0-2longitudinalseptaand1-6transversesepta,andvariableinshape andsize,mostlyovalshapewithrudimentarybeakandinsizemeasuringabout10.26-77.52 x 4.56-14.82 µm. Based on the morphological characters, the organism was identified as *AlternariaAlternata***(Keissler, 1912; Barnett and Hunter, 1973)** (Plate 1).

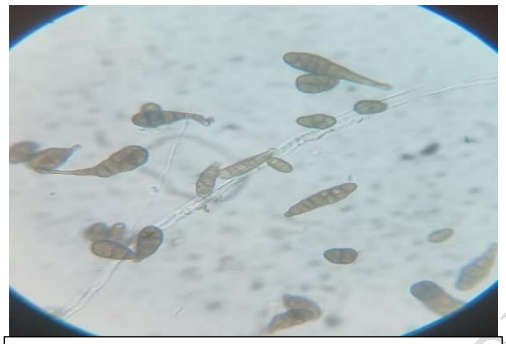


Plate1.Conidiaof*Alternaria alternata* (45X)

2.1.4 BiocontrolAgent*Trichodermaharzianum*

Thebiocontrolagent*Trichodermaharzianum*wasobtainedfromLaboratory,Departmentof Plant Pathology, Naini Agricultural Institute, Sam Higginbottom University of Agriculture, Technology and Sciences, Prayagraj, U.P. *Trichoderma harzianum* was sub-cultured ~~in~~ *in vitro* for antagonism test against *Alternariaalternata*(Plate 2).

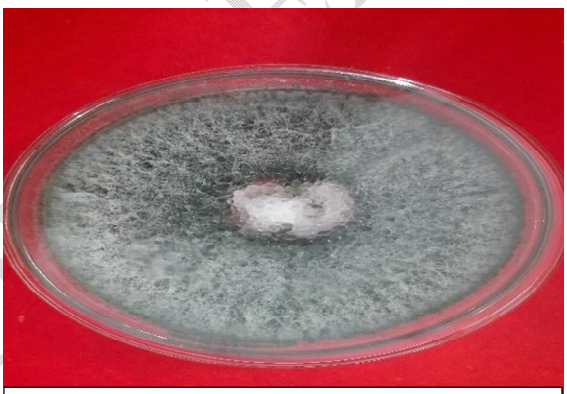


Plate2.PureCultureof*Trichoderma harzianum*

2.2 Methods

Dualculturewasperformedtochecktheantagonismabilityof*Trichodermaharzianum*against *Alternariaalternata*for mycelial inhibition and overgrowth. This was done following six differentdesignsofdualculturetechniquetoobservetheefficiencyof*T.harzianum*against*A. alternata*andcontrolplateswerealsoset.*T.harzianum*and*A.alternata*wassub-culturedonto PDAfor7days.Themarginofthecolonywascutwithsterilecorkborerandwasplacedin90 mm diameter Petri plate containing PDA in different designs **(Nafiza *et al.*, 2021)**.

2.2.1 DesignsofDualCulturePlates

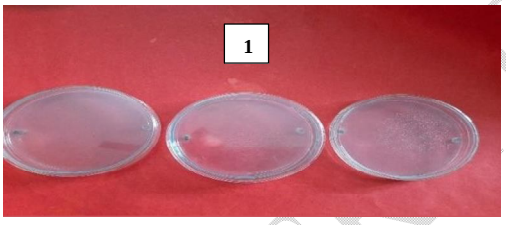
In first design, one disc of *A. alternata*was set against one disc of *T. harzianum*. In second design,twodiscsof *T.harzianum*wereseton theperipheryofbothsidesofthePetridish and one disc *A. alternata*was set at the ~~centre~~center of the Petri dish. In third design, three discs of *T. harzianum* were set on the periphery of the Petri dish surrounding one disc of *A. alternata*on the ~~centre~~center of the Petri dish. In fourth design, four discs of *T. harzianum* were set on the peripheryofthe Petri dish surrounding onediscof *A.alternata*on the~~centre~~centerofthePetri dish. In fifth design, five discs of *T. harzianum* were set on the periphery of the Petri dish surroundingonediscof *A.alternata*onthe~~centre~~centerofthePetridish. Insixthdesign,sixdiscs of *T. harzianum* were set on the periphery of Petri dish surrounding one disc of *A. alternata*on the~~centre~~centerofthePetridish.Incontrolplate,onlyonediscof*A.alternata*wassetonthe~~centre~~center of the Petri dish (Plate 3).

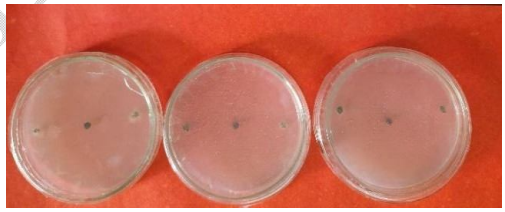
Each design of dual culture technique was replicated three times. All the plates are incubated at28±1°Candantagonisticactivitywastested7daysafterincubationbymeasuringtheradius ofthe*A.alternata*colony(R2)inthetreatmentplatesandtheradiusofthe *A.alternata*colony inthecontrolplate(~~R1~~GI)atregularintervals.Theabilityof*T.harzianum*toovergrowthecolony of *A. alternata*was observed and compared with the control treatment.

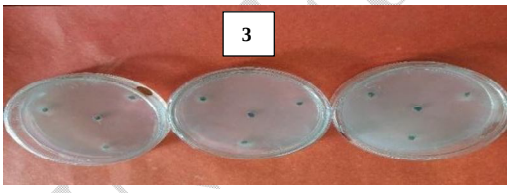
~~Theinhibition levels werecalculated usingtheformula~~ **~~$[(R_1-R_2)/R_1] \times 100$.~~**

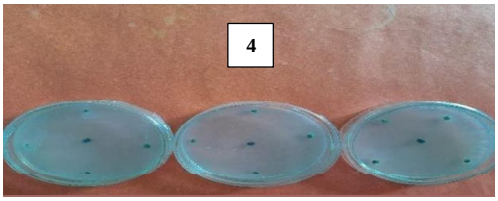
the growth inhibition percentage (GI %) for each treatment was calculated according to Arora and Upadhay (1978) as follows: GI%= ((C-T) /C) *100 where, GI% = percent of growth inhibition over control, C = radius growth of control (mm), T = radius growth of ***Trichoderma***(mm).

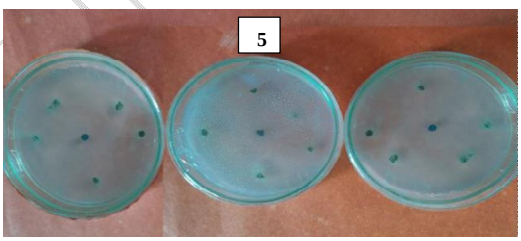
2

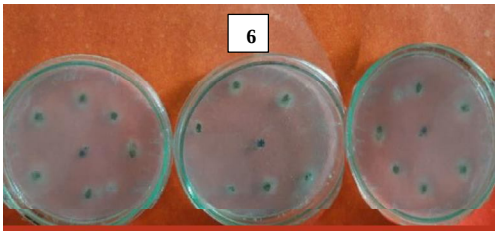












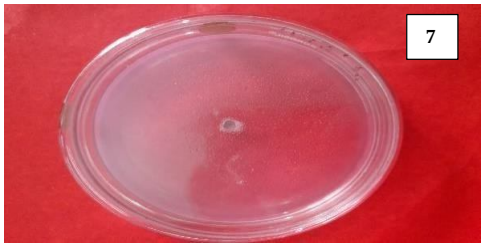


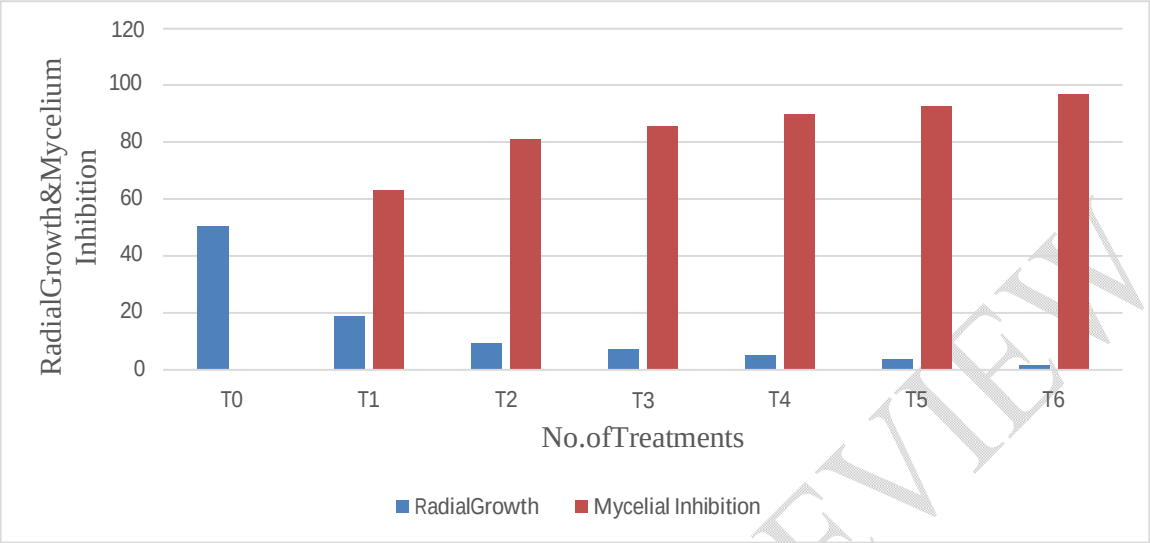
Plate 3. Different designs of dual culture plates containing *Trichoderma harzianum* and *Alternariaalternata*inPDA;1-onediscof*A.alternata*wassetagainstonediscof*T.harzianum*, 2- two discs of *T. harzianum* were set on the periphery surrounding one disc of *A. alternata* on thecentreofthePetri dish, 3-threediscs of *T. harzianum* wereset on theperipherysurrounding one disc of *A. alternata* on the centreof the Petri dish, 4- fourdiscs of *T. harzianum* wereset on theperipherysurroundingonediscof*A.alternata*onthecentreofthePetridish,5-fivediscsof *T. harzianum* wereset on the periphery surrounding one disc of *A. alternata* on the centreof the Petri dish, 6- six discs of *T. harzianum* were set on the periphery surrounding one disc of *A. alternata* on the centre of the Petri dish, 7- control plates of *A. alternata*.

3.RESULTS&DISCUSSION

Table1.Radialgrowthandmycelialinhibitionindualculturetechniqueusing *Trichodermaharzianum*against*Alternariaalternata*

Treatment Notation	Treatmentdetails	Radial growth (mm)	Mycelial inhibition (%)
		7 DAI	
T_0	Only onedisc of*A. alternata*	50.33[a]	-
T_1	One disc of *T. harzianum* against one disc of *A. alternata*	18.50[b]	63.24
T_2	Two disc of *T. harzianum* against one disc of *A. alternata*	9.50[c]	81.12
T_3	Threediscof*T.harzianum*againstonedisc of *A. alternata*	7.25[d]	85.59
T_4	Four disc of *T. harzianum* against one disc of *A. alternata*	5.00[e]	90.06
T_5	Fivediscof*T.harzianum*againstonedisc of*A. alternata*	3.67[e]	92.70
T_6	Sixdiscof*T.harzianum*againstonediscof *A. alternata*	1.66[f]	96.70
$CD_{0.05}$		**1.43**	

Figure1.Graphicalrepresentationofefficacyof*Trichodermaharzianum*against *Alternariaalternata*indualculture~~tecghnique~~technique at7DAI



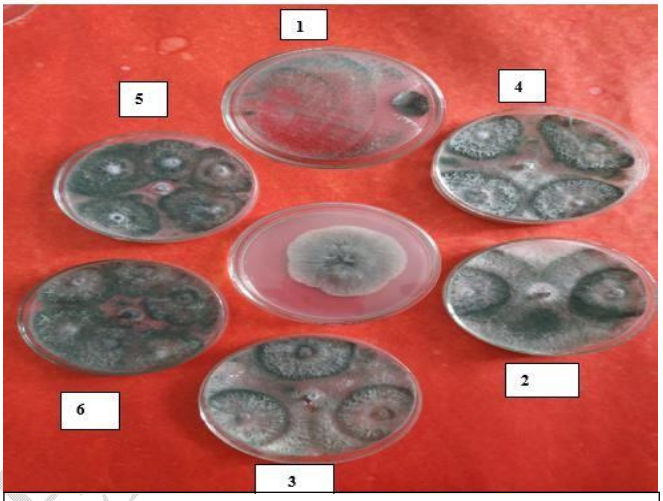


Plate 4. Plates after 7 DAI(1- one disc of Aa with Th, 2- one disc of Aa with two discs of Th, 3- one discofAawiththreediscsofTh,4-onediscofAa with four discs of Th, 5- one disc of Aa with five discs of Th and 6- one disc of Aa with six discs of Th.

Th-*Trichodermaharzianum*,Aa-*Alternariaalternata

All six designs have shown reduced growth of *Alternariaalternata*in the dual culture plates when compared with the control plate. Among the six designs of our experiment, the sixth design where six discs of *Trichoderma harzianum* was set on the periphery of the Petri dish surrounding *Alternariaalternata*on the ~~centre~~center have shown maximum mycelial inhibition of 96.70%,wheretheradialgrowthof*Alternariaalternata*was1.66mmfollowedbyfifthdesign wherefivediscsof*Trichodermaharzianum*weresetontheperipheryofthePetridish

surrounding one disc of *Alternariaalternata*on the ~~centre~~center of the ~~Petridish~~Petri dish where the radial growth of *Alternariaalternata*was 3.67 mm, fourth design where four discs of *Trichoderma harzianum* were set on the periphery of the Petri dish surrounding one disc of *Alternariaalternata*on the ~~centre~~center of the Petri dish where the radial growth of *Alternariaalternata*was 5mm.Thefirst,secondandthirddesignrecordedradialgrowthof*Alternariaalternata*of7.25 mm, 9.50 mm, 18.50 mm respectively. In control condition, radial growth of *Alternariaalternata*was 50.33 mm after 7 days of incubation (Table 1). Mycelial overgrowth of *Trichodermaharzianum*wasalsoobservedinthedualcultureplatesafter7daysofincubation (Plate 4).

The probable reasons for such findings may be due to the secretion of extracellular cell degradingenzymesandproductionofsecondarymetabolitesby*Trichodermaharzianum*which inhibited the growth of the *Alternariaalternata***Zade*et al.* (2018)**. The results of the present findingsagreewith **Nafiza*etal.* (2021)**whocarriedoutdual culturewith different numberof discs of *Trichoderma harzianum* against *Magnaportheoryzae*and recorded maximum inhibitionofpathogenintheplatewithmaximumnumberofdiscsof*T.harzianum*setagainst thepathogen.Similarfindingswerealsorecordedby**Khaliquee*tal.*(2016)**whoalsoobserved maximum inhibition of *A. alternata*when *T. harzianum* was applied from four sides keeping one disc of pathogen at the ~~centre~~center.

5. Conclusion

Sixdifferentdesignsofdualculturetechniquewith*Trichodermaharzianum*against*Alternariaalter nata*was used. *In vitro* analysis revealed that the sixth design where six discs of *Trichoderma harzianum* was set on the periphery of the Petri dish surrounding *Alternariaalternata*on the centre have shown maximum mycelial inhibition of 96.70%. All the designs of dual culture technique were found effective *in vitro* test against *Alternariaalternata*.

References

Arora, D. K. and Upadhyay, R. K. (1978).Effect of fungicide staling growth substances on colony interaction. Pl. & Soil, 49: 685-690.

Barnett,H.L.andHunter,B.B.(1972). Illustratedgeneraofimperfectfungi.Burgess Publication Company. Minnesota, USA.

Chirutala, N., and Simon, S. (2021). Use of bio-resources in the management of *Alternariaalternata*and *Rhizoctoniasolani*of stevia (*Stevia rebaudiana*).

Dhange, P. R., Srinatha, T. N., and Kandpal, K. (2023). Stevia farming: a moneymakingventure for farmers. *Indian Farming*. 73(12):19-22.

Ferreira,F.V.andMusumeci,M.A.(2021).*Trichoderma*asbiologicalcontrolagent:scope andprospectstoimproveefficacy.*WorldJournalofMicrobiologyandBiotechnology*.37: 90.

~~**Hariharan, G., Rifnas, L. M., and Prasannath, K. (2022).** Role of *Trichoderma* spp. in biocontrol of plant diseases. In *Microbial biocontrol: Food security and post harvest management* (pp. 39-78). *Springer, Cham*. https://doi.org/10.1007/978-3-030-87289-2_3.~~

Keissler,K.V.(1912). ZurKenntnisderPilzfloraKrains.*BeihefteZumBotanischen Centralblatt.* 29: 395-440.

Khaliq,A.,Khan,H.,Maria, N.,Khan, N.,Zada, L., Ali, M., Zahoor,M. andSamiullah. (2017).*Invitro*efficacyof*Trichodermaharzianum*Rifaiagainstvariousisolatesof*Alternaria* sp. *Journal of Entomology and Zoology Studies*. 5(1): 442-445.

Kumar,R.,Sharma,S.andSharma,M.(2014). Growthandyieldofnatural-sweetenerplant-stevia as affected by pinching. *Indian Journal of Plant Physiology.*

Nafiza, Z., Aminuzzaman, F.M, Laila, L. and Ashrafi, M.L. (2021). Comparative *in vitro* efficacy assessment methods of a bioagent*Trichoderma harzianum* THR 4 against rice blast pathogen *Magnaportheoryzaeoryzae*. *Open Acess Library Journal*. 8:e7510.

Nayak,A.M.,Dhange,P.R.,Farooqkhan,andIsmayil,M.S.M.(2023). Fungalbioagents andbotanicalsefficacyagainst*Alternariaalternata*responsibleforleafblightdiseaseof*Stevia rebaudiana. International Journal of Plant & Soil Science*. 35(22): 254-260.

Rahman, M. A., Rahman, M. A., Moni, Z. R. and Rahman, M. A. (2020). Evaluation of bio-controlefficacyof *Trichoderma*strains against *Alternariaalternata*causingleafblightof Ashwagandha [*Withaniasomnifera*(L.) Dunal]. *Journal of Forest and Environmental Science*.36(3): 207-218.

Rashid,Z.,Ahngar,T.A.,Nazir,A.,Dar,Z.A.,Khuroo,N.S.,Majeed,S.,Naseer,S., Bashir, S., Rakshanda,A., Iqbal, S., and Jan,S. (2021). Research technology of Stevia. *Cutting-edge research in agricultural sciences* (Vol. 6).

Roco, A., Castañeda, P., and Pérez, L.M. (1993). Oligosaccharides released by pectinase treatment of Citrus limon seedlings are elicitors of the plant response. *Phytochemistry*. 33:1301-1306.

Toussoun,T.A.,andNelson,P.E.(1976).*Apictorialguidetotheidentificationof Fusarium species* (2nd ed.). University Park, PA: Pennsylvania State University Press.

Zade, S. B., Ingle, Y. V., and Ingle, R. W. (2018). Evaluation of fungicides, botanicals and bio agents against *Alternariaalternata*incitant of leaf spot of soybean. *Journal of Pharmacognosy and Phytochemistry*. 7(5): 1687-1690.