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ISOLATION AND CHARACTERIZATION OF PECTINASE FOR INDUSTRIAL APPLICATION

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ABSTRACT

Microorganisms, such as bacteria, are useful organisms in degrading the dead organic material, hence recycling the compounds. They are also useful in the production of various commercially important enzymes. The soil samples from different regions of Islamabad and Rawalpindi were taken for the investigation of bacterial strains that produce pectinase enzymes. Soil samples were diluted and streaking was carried out on the nutrient agar media for the isolation of different bacterial strains. The pH of nutrient agar media was adjusted at 8 and the cultures were incubated at 37^{o}C for 36 hours. Isolated cultures were tested on screening media for the investigation of production of pectinase by that isolated bacterial strains. Enzyme was harvested and centrifuged. Supernatant was taken as crude extract while the bacterial cells were pellet down. Crude extract was subjected to the Lowry assay and the total protein content was recorded.Ammoniumsulphateprecipitationwascarriedoutforthepartialpurification of pectinase enzyme. The results of Lowry assay shows that the protein content was

decreased from crude extract to the purified protein. The protein content obtained after column chromatography was 0.099mg/ml. The activity of enzyme obtained after the purification processes was 12.03 Units. Protein wapurified 3.2 folds and 57%yieldwas obtained.

Keywords; Enzyme, Pectinase, Gel chromatography, Industries

INTRODUCTION\

The nature has provided the microbes with enormous abilities. One of the great qualities of microbes is the production of enzymes which are used by man over a long time. Certain microorganisms, i.e., bacteria and fungi, are able to produce pectinase enzymes which are of remarkable importance in industrial as well as in commercial sector (Jayani et al., 2005). Pectinase are about to dominate the industrial sectors of food, paper and textile as they are able to degrade pectin present in cell wall of dicots (Kashyap et al., 200) Pectinaseenzymesarediversegroupofenzymesthatareabletohydrolysepectic substances mainly from the plant sources. Many species of fungi and bacterial strains produce abundantly these pectin hydrolyzing enzymes(Pedrolli et al., 2009). Pectinases are responsible for the extension of cell wall so they are one of themost important enzymes for plants (Jayani et al., 2005), and are also responsible forthe softening of the plant tissues while they get mature (Sakai, 1992; Aguilar and Huirton, 1990).

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It is a common practice that pectinases are utilized for degradation of various plant residues, extraction of juices from apples and other citric fruits as well. These enzymes have also been utilized for fermentation in brewing industries since 1960s (Semenova et al., 2006). Pectinases are efficient enough to degrade acids of pectin, i.e., homogalacturonan and rhamnogalacturonic acid, and convert them into useful compounds like sugars. Pectinase enzymes are of great industrial importance as they hydrolyse the pectic substances present in fruits into useful products. On the otherhand,pectinasesaretheprimaryenzymesthatareinvolvedinspoilinganddecayingofprocessed food (Barth et al.,2009). Cellwallisadistinguishingcharacterofplantwhichgivesthemprotectionand is of vital importance. Due to immobile nature, plants have to face and with stand every type of harsh environmental conditions and attack of pathogen and herbivores.Thediversityofchemicalconstituentofcellwalllikeproteins,polysaccharides

aromaticandaliphaticcompoundsallowthemtoflourishindifferentenvironmentalniches(Mohnen, 2008; Reddy and Saritha , 2016; Riou et al., 1992).

Asweknowstructuralconstituentsofplantcellwallarecellulose,hemicellulose and pectic substances. Pectic substances are polysaccharide in naturewithhighmolecularweightandnegativecharge(Osborne,2004).Structurallytheyare having central chain in which the units are linked in α-1-4 glycoside linkagepartiallyesterifiedwithmethylgroupsaswell.Thesemethylatedmoleculesareknownaspectinwhiledemethylasemoleculesarecalledpecticacidorpolygalacturonicacid.

Pectic substances are compounds with very high molecular weight. These arefoundinmajorproportioninthecellwallofplantsparticularlymiddlelamellae,which is the thin wall between demarcating adjacent cells.In plant tissues, theirfunctionis to give them structural integrity andcohesion (Rombouts et al., 1980).

Pectinasesare usually classifiedasacidic pectinasesandbasicpectinases.Acidic pectinases are used for fruit juice and wine preparation. Acidic pectinases areused to remove suspended matter and as a result sparkling clear juice is obtained.Ahighly viscous juice is obtained when pectin-rich substances are crushed mechanicallyand stay linked to the pulp. Thus, a gelatinous structure of pectin tends to halt or obstruct the process of juice extraction (Khatri et al., 2015). While basic pectinases are used in paper, textile and pulp industry (Sharma and Satyanarayan,2004).

Pectin is a homopolysacharide in structure mainly present majorly in the cellwall of plants. That is why, it makes almost one third dry weight of the different planttissues (Gupta et al., 2008). Pectin is a matrix of jelly like substance which work as a cementing agent between plant cells of dicots, so that other cell wall component like cellulose fibrils are embedded in it (Braconnot et al., 2006). Pectin is commonly available as a white to light brown powder used to stabilize fruit juices, gelling agent and as a source of dietary fibrein energy drinks.

In order to overcome the energy crisis in the world, attention is focused on the renewable energy resources, like forest and agriculture remains, which mainly contain cellulose, lignin, xylan and pectin. Due to abundance of these resources, they aregainingalotofattentionasalternativeformoffeedstockandenergy.Severalmicrobes by producing vast array of enzymes can utilize such substances and carbonas energysources in variety of habitats (Sethi et al., 2016).

APPLICATIONSOFPECTINASES

According to the available physical conditions the application of pectinolyticenzymes is varied. Pectinases has been utilized in various industrial applications over the years, such as processing of fibre, tea, extraction of oil, textile processing, for purification of industrial waste water which contain pectinaciouces material. They arereportedtohaveroleinpurificationofvirusesandinpapermaking.Pectinaseenzymes were commercially used by man. In past. Pectinasesinspiteoflargescaleapplicationinvariousindustrialapplications,theyhaveaprimary and major role in fruit processing industry. They are used to enhance juiceyield, for giving colour and also as juice clarifier by decreasing the viscosity of fruit juices (Junweietal.,2000). They are use dinpressingandstrainingstagesinprocessingofcertainfruitslikeapple,pearandgrapes.Detailofsomeoftheapplicationofpectinasesis given in the following section.

Paper and Pulp Industry

Pulp and paper industries are now using various productsincluding enzymesto ease their problems in manufacturing process. Pectinases produced by *Bacillus sp.*and *Erwiniacrotovora* (Pedrolli et al., 2009),hasbeenused for obtaining bast fibers from *Edgeworthiachrysantha* due to its strong softeningactivity (Tanabe and Kobayashi, 1987). Japanese paper has been produced by using this retted bast (Horikoshi, 1990). Moreover, pectinases can also depolymerize pectinin order to lower the cationic demand of pectic solutions and filtrate form peroxidebleachingresultingin thebetter production of paper.

Textile Processing

The ability toremovesizingagentsfromcottoninanefficientandnon-hazardouswayledtotheuse of Pectinase in conjunction with other important enzymes like amylases, lipases, cellulase and hemicelluloses, thus replaced causticsoda which is very toxic to the environment and was used earlier. Moreover, they canbe used in the process of Bio-scouring; a process in which non-cellulosic materials areremoved from the fibre without causing any damage to the fibre in a safe manner (Jayani et al., 2005).

Waste Water Treatment

As we know waste water is released from many food processing industries. Wastewaterfromthecitrusprocessingindustrieswhichlargelycontainsperteinaciousmaterials is barely decomposed by the microbes in a treatment called activated-sludge treatment. Endo-pectately as secreted by as oft-rotpathogen *Erwiniacrotovora*hasbeen reported to be useful in the pretreatment of pectenocius water produced from vegetable processing industries(Tanabe et al., 1986).

Purification of Plant Virus

Alkaline pectinases and cellulose digesting enzymes can be used to give very purepreparation of viruses in those cases when the virus particle is restricted to the phloemtissue (Jayani et al., 2005).

MICROORGANISMSANDPECTINASEPRODUCTION

Primary source of industrial enzymes are microorganisms, as 50% are fromthe fungi and yeast, 35% coming from the bacteria and remaining 15% are from theplants.Filamentousmicrobesarewidelyusedforsubmergedandsolid-statefermentation as these have the prime advantage over the non-motile bacterial andfungal species (Smith, 1987). Mehta et al., (1992) reported that they isolated 168differentspeciesofbacteriafromsoilvegetablesourcesandreportedthatthesespecies have the ability to use pectin as only source of carbon. Out of which 30% had shows significant pectinase digesting ability.

Submerged fermentation process is generally used to produce the pectinasefrom microorganisms. The process involves the dissolution of the substrate in waterand the inoculum of bacteria is allowed to grow in the liquid media. Large volume ofdistilled water is required to produce the enzymes in submerged fermentation and also requires constant shaking (Geetha et al., 2012).

Pectinases used in fruit industry are produced onlarge scale from Fungi as they are the most efficient in this process especially the *Aspergillus sp*. Moreover,theyhaveanotheradvantagethatthepHofenzymesproducedbyfungiisveryclose to the pH of fruit juices (Ueda et al, 1982). However, for the preparation of vegetablepurees requiring pH in neutral range, they cannot be used (Chaeson et al., 1978).Moreover,fungalenzymepreparationsarerelativelylessstableathighertemperature s, so maceration should be carried out at temperature not exceeding 45^{o}C.This limitation, necessitate that pasteurizing step should be carried out to inhibit thegrowthofmesophilic microbes (Silley, 1986).

Among bacterial species *Bacillus sp., Coccisp, Pseudomonas sp., Erwinia sp.*and *Chryseobacterium sp.* are the pectinase producing microorganisms. (Roy et al.,2018 Karthik, Kumar and Rao, 2011; Kumar and Sharma,2012). Bacillus sp. is the major contributor in the production of alkaline pectinaseenzymes(Mohandas et al.,2018).

MATERIALS AND METHODS

In order to investigate the pectinase producing bacterialstrains, researchwascarriedoutinEnvironmentalandIndustrialBiochemistryLaboratoryinBiochemistryDepartment, PMAS-Arid Agriculture University Rawalpindi.

Collection of soil samples

In order to isolate the bacterial strains from soil, different soil samples werecollectedfromdifferentregionsofIslamabadandRawalpindiforthemicrobiological investigation of pectinase producing bacterial strains. Soils were collected from surface and at a depth of 15 centimeter and both samples were thoroughly mixed to obtain a composite sample. Soil samples were collected in sterile plastic containers. Solid waste such as broken bottles and remains of plastic bags were removed and the composite sample was passed through a2mmmesh.

Preparation of nutrient medium

Nutrient Agar (NA) medium was prepared by adding 14g of nutrient agar in500ml of sterile distilled water. It was boiled to homogenize the solution. After thesolutionwashomogenized,thepHofNAmediumwasadjustedat8andthenitwas autoclaved. Autoclaved nutrient agar medium was poured in sterile petri platesand they were remained in Laminar Flow Cabinet (LFC) for 24 hours for the detection of contamination.

Isolation of bacteria

Soilsampleswerepouredinsteriledistilledwater.Tenfolddilutionsofeach sample were made in ten sterilized test tubes. Samples were picked withsterile cooled loop from the dilutions and streaked in 4 quadrants on Nutrient agarmedium.Streakingwascarriedoutusingstreakplatemethod.Afterstreaking,thesesamples were incubated at 37^{o}C for36 hours.

Sub culturing of bacteria

Mixed bacterial colonies were developed onNAmedium.Toobtainthepurecultures,themorphologicallydifferentcoloniesweresubcultured on NA medium and were incubated at 37ºC for36 hours.

Preparation for of screening media

Pectin Screening Agar Media (PSAM)wasusedtotesttheactivityofbacterial isolates. PSAM was prepared according to the following composition:0.3%$(NH_4)_2HPO_4$,0.2%KH_2PO_4,0.3%K_2HPO_4,0.01%$MgSO_4$,2.5%Agarand
1%Pectin (Beulah et al., 2015) as shown in Table 1

PSAM was boiled to obtain a homogenized solution. The pH of PSAM wasset to 8. After that it was autoclaved. Autoclaved PSAM was poured in sterile petri plates and left in BSC for 24 hours to observe the contamination.

Table1. Composition of Screening Media

Sr.No.	Chemicals	Quantity
1	$(NH_4)_2HPO_4$	0.3%
2	KH_2PO_4	0.2%
3	K_2HPO_4	0.3%
4	$MgSO_4$	0.01%
5	Agar	2.5%
6	Pectin	1%

SCREENINGOFBACTERIAL STRAINS

Screening of bacterial strains was carried out by spot inoculation of all thebacterialisolates on PSAM (Rokade et al., 2015).

Spotinoculation wasinvolved only the touch of bacterialisolates on PSAM. All the spot inoculated petri plates were incubated at 37ºC for 36 hours in order toob serve their pectinase digesting ability. The bacterial strains showing the positive pectinase activity were able to develop a colony and formed clearance zones by utilizing pectin as a sole source of carbon. Such bacterial strains were picked up as positive cultures.

PURIFICATIONANDPRESERVATIONOFCULTURES

Bacterial strains that showed the positive pectinase activity were picked and streaked on NA medium and incubated at 37ºC for 36 hours. These pure bacterial strains were stored at 4ºCforfurther use.

MORPHOLOGICALCHARACTERIZATIONOFBACTERIA

Gram Staining

In order to characterize the bacterial strains used in the study on the basis oftheir Gram staining, two drops of distilled water were taken on a glass slide with asterileloop.Asmallsampleofbacterialcolonywaspickedandgentlystirredonthe water drop and stirred gently. Then smear was allowed to air dry. After thesmear on theglass slide was air dried, slide was passed through the flame of Bunsen burner three times togetthe smear heat fixed. After the settlement of smear, slide was flooded with crystal violet and left for 1 minute. Then it waswashedandiodinewasappliedonitandagainleftfor1minute.Afterthatdecolorizer was applied for 10 seconds and immediately rinsed with water. At theend safranin was flooded for 40 seconds and washed. The slide was left to air dry.The slide was observed using a light microscope under oil-immersion. The purplestained colonies were Gram positive bacteria while the colonies that appeared pinkwereGram negative bacteria.

Motility of Bacterial Strains

Lightmicroscopetechniquewasusedinordertoinvestigateaboutthecellshape and motility of bacterial cells. Hanging drop technique was used to examinethe motility of bacterial cells. Paraffin ring was applied to the glass slide to make acircular concavity. A toothpick was used to dab Vaseline around paraffin so thatcover slip can get attached on glass slide. A loop of deionized water was added onthecoverslipandthenbacterialcolonywasmixedwithwateronthecoverslip.The glass slide was inverted over the drop by turning upside down on the cover slipand the Vaseline sealed the cover slip over the concavity. This prepared slide wasobservedunderlightmicroscope.Theedgesoftheconcavitywerefocusedcarefully.

ENZYME PRODUCTION

InoculumPreparation

Bacteria that gave the positive pectinase activity on PSAM were grown inliquidmedium.PectinBroth(PB)wasusedforthepurposeofinoculumpreparation.PBc ontained2.5%pectinasacarbonsourcedissolvedinsteriledistilledwater.

3.1.1 PreparationofProductionMedia

Citruspectin,obtainedfromorangepeels,wasusedasamajorsourceofcarbon. Production media was prepared according to the following composition:2%$NaNO_3$, 1%KH_2PO_4, 0.5% KCl, 0.5% $MgSO_4$and 5% Pectin (Kalaichelvan,2012).Table3.2.

Pectin was used as a carbon source. All these components were dissolved insteriledistilledwaterandboiledtohomogenizethesolution.ThepHoftheproduction media was set at 8. Production media was autoclaved and was used forfermentation.

Fermentation

Bacteria obtained from the inoculums media were used for fermentation to

produce pectinase enzyme. 2ml of inoculum was poured in 250ml of productionmedia. Then it was placed in rotatory shaker with a speed of 120rpm at 37^{o}C for 48hours.

Total Protein Determination

The total protein content in the crude extract was determined by using Lowry assay taking Bovine Serum Albumin (BSA) as standard. 2% sodium carbonate(Na_2CO_3) and2N sodium hydroxide (NaOH) were mixed together to form Solution

A. 1% solution of copper sulphate pentahydrate ($CuSO_4.5H_2O$) was prepared indistilled water. 1% solution of copper sulphate was called Solution B. Solution Ccomprises of 2% sodium potassium tartarate ($KNaC_4H_4O_6.4H_2O$) in distilled water.SolutionA,BandCweremixedtogetherinaratioof48:1:1respectivelytoforman alkaline solution. 100µL of extraction buffer was added in 100µL of BSA andsampleseparately.700µLofalkalinesolutionwasaddedtoalldilutionsofBSAand sample to make the total volume up to 900µL. After the addition of alkalinesolution, the dilutions of BSA and samples were incubated at room temperature for20 minutes. After incubation, 100µL of Folin's phenol reagent was added. Afterfollowing the standard protocol of Lowry assay, the samples were subjected to spectrophotometer.

Ammonium Sulphate Precipitation

Inordertopurifytheprotein,thefirstprocessthatwasfollowedwasammonium sulphate [$(NH_4)_2SO_4$] precipitation. This technique purified the proteininthecrudeextractpartially.Precipitationmakesiteasyforproteintogetconcentrated and partially purified. Serial precipitations were carried out using varying amounts of ammonium sulphate from 50% to 90% by a difference of 10%.Ammonium sulphate was gradually added and gently stirred to get dissolved. Thenitwasallowedtostirat4^{o}Cfor2hours.Aftertheammoniumsulphatewascompletelydissolvedincrudeextract,itwascentrifugedat10000rpmfor15minutes at 4^{o}C (Sethi et al., 2016). The serial fraction that was able to produce more pellets was selected for further precipitations. The supernatants and pellets of all ammonium sulphate fractions were analyzed by enzyme assay. Thefractionwhichcontainedmoreenzymeconcentrationsinpelletsandleastenzymein supernatant was selected for further precipitations. In further precipitations, thesupernatant was discarded and pellet was dissolved in a phosphate buffer of pH 5.6.Theconcentration of partially purified protein was determined by Lowry assay.

Dialysis and Concentration

Phosphate buffer containing the dissolved pellet was taken into the cellulosedialysis tubing againstdistilled water.Itwas allowed to stir for24 hours. Waterwaschangedoccasionally.ThedialysatewasfurtherusedinLowryassayforthe

Determination of protein content.

Gel Filtration Chromatography

In order to purify the pectinase enzyme from other proteins present in the sample, gel filtration chromatography was carried out. A glass column of 20cmlength having 1.5cm diameter was used to carry out gel filtration chromatography. The length of gel bed was 12cm. 1g Sephadex G-100 was taken in 100 ml of sterile distilled water and this mixture was left for overnight. Then it was used for packing of column up to 50cm. The packing of gel column was followed by phosphate buffer. Settlement of gel was carried out at 2ml/minute. After the gel bed was formed, 1 ml of sample was applied to column and sample flowed down with passage of time. 35 eluted samples were taken of 1ml each. After the samples were collected in the eppen drof of tubes, they were followed by enzyme assay to find out the activity.

Determination of Enzyme Activity

Dinitrosalicylicacidassay(DNSA)wasusedtoobservetheactivityofpectinase. 100µL cell free supernatant was incubated with 100µL of substrate. Thesubstrate used was 1% citrus pectin. The mixture of supernatant and substrate wasincubated at 40^{o}C for 10 minutes. After that 400µL of DNSA was added and boiledfor 15 minutes. Distilled water was added to dilute the solution up to 5ml.Using the spectrophotometer, absorbance was measured at 530nm.

RESULTS AND DISCUSSION

Pectinases are one of the most essential enzymes that are used widely in textile, juice and paper industries. Bacteria and fungi are used to produce pectinases. These enzymes degrade pectin which is the most important part of secondary cell wall ofplants.Pectinasesarecategorizedintoprotopectinases,esterasesanddepolymerases.This classificationisbasedontheirwayofdegradingpectinmolecule(Sharma et al.,2012).

Pectinases were produced by submerged state fermentation in which citrus peels were used as a carbon source. Bacteria were isolated from the soil samples.MorphologicallydifferentcoloniesweresubculturedonNA.Eachisolatewassubjected to screening media. Bacterial cultures showing positive pectinase activitywere inoculated in the fermentation media. Enzymes produced through submerged state fermentation were subjected to different assays.

ISOLATIONANDSUBCULTURINGOFBACTERIALSTRAINS

DifferentbacterialstrainswereisolatedinIndustrialandEnvironmentalBiochemistryLab,inUniversityInstituteofBiochemistryandBiotechnology(UIBB), PMAS-AridAgriculture University Rawalpindi.Tenfold serialdilutionsof soil samples were prepared. Bacteria were picked from the homogenized soilsample with a sterile cooled loop and streaked on NA medium. Petri plates wereplacedinincubatorat37^{o}Cfor36hours.After36hours,culturesofdifferent

bacterialstrainsweregrownonNAmedium.Morphologicallydifferentbacterialstrainsweresubculturedon NAmedium to obtain the purecultures.

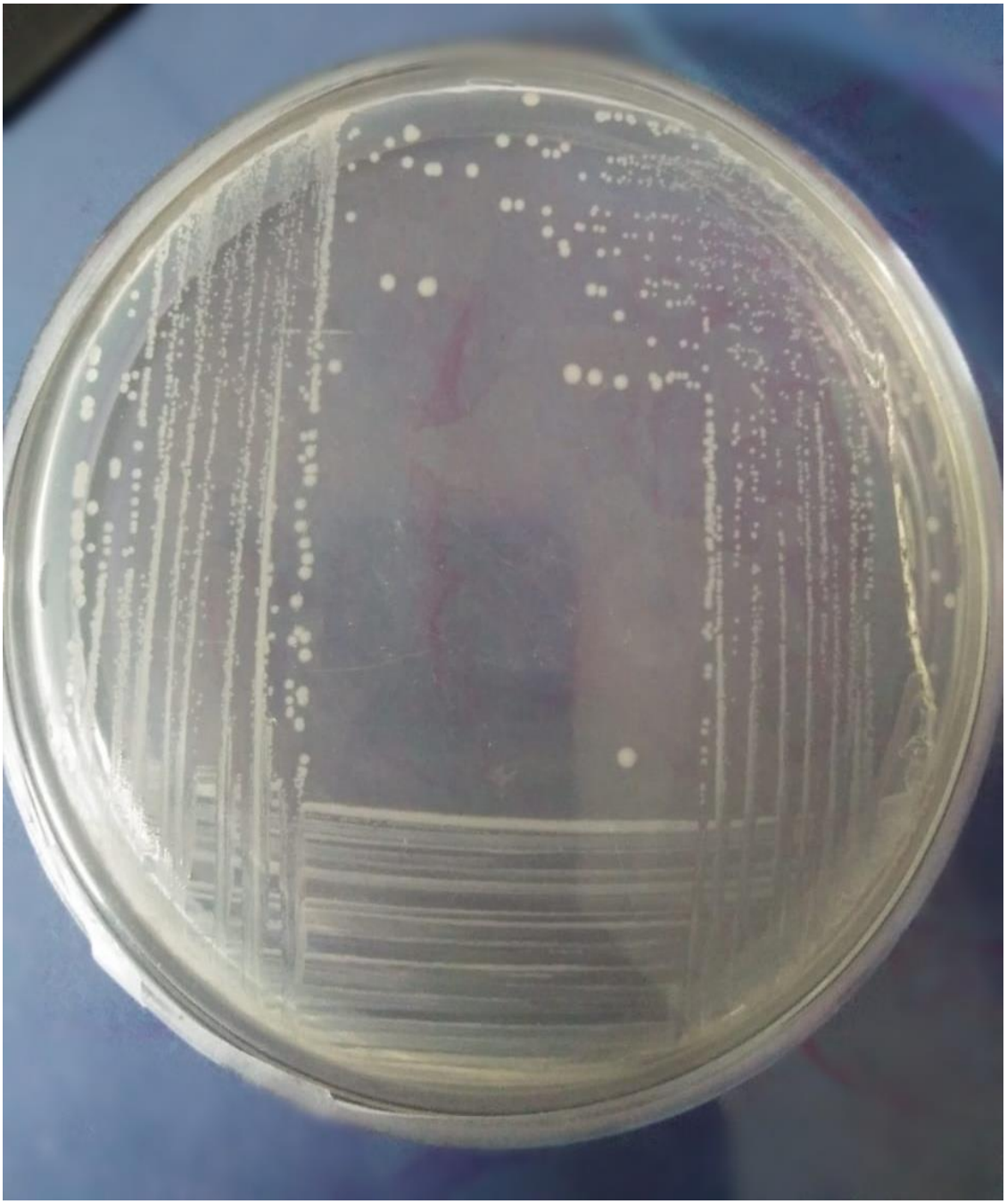

Figure 1. Subculturing

SCREENINGOFBACTERIALSTRAINS

PSAMwasusedtoinvestigatetheactivityofbacterialisolates.Spotinoculation was carried out for this purpose. Bacterial strains that were able toproduce pectinase enzyme utilized the pectin as a sole carbon source and formed aclearance zone around their colonies. Clearance zone was observed by flooding thescreening plates with iodine-potassium iodide solution. Clearance zones of average2.06cmwere formed on thescreeningmedia.

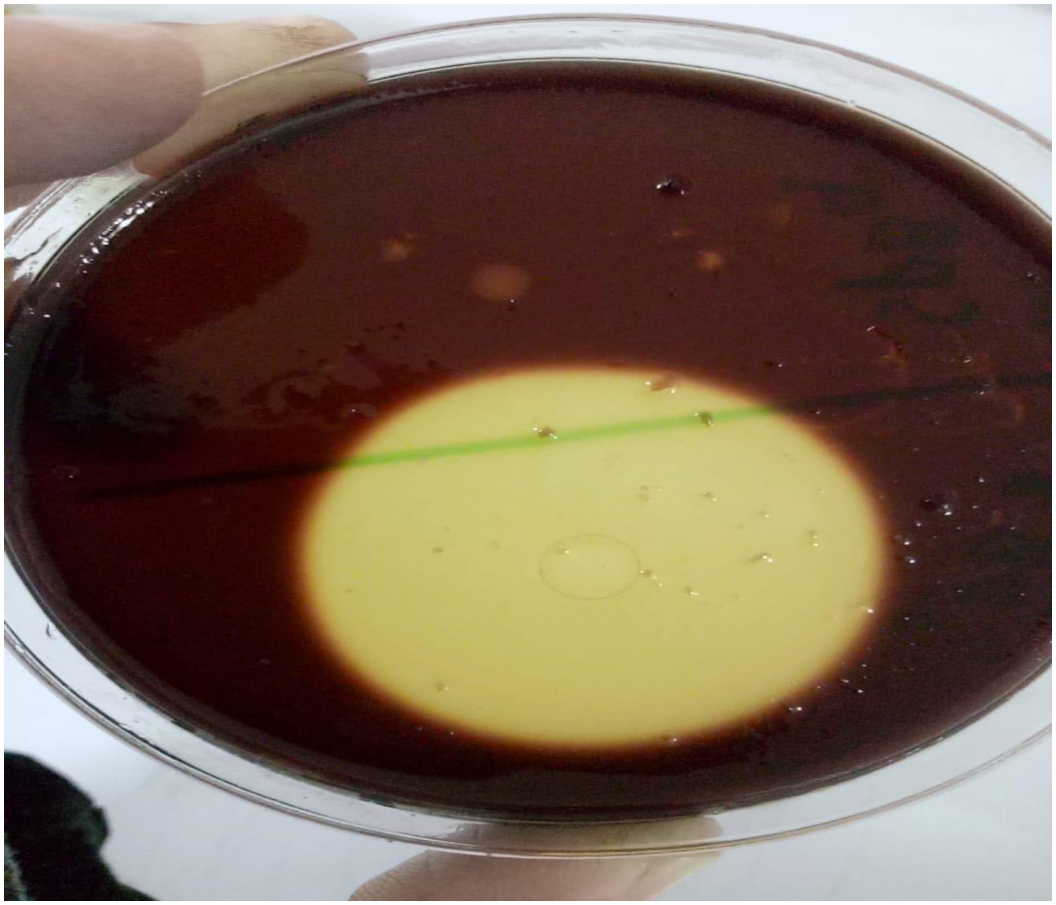

Figure 2:Clearancezoneformed byBacterial Colony

MORPHOLOGICALIDENTIFICATIONOFBACTERIALSTRAINS

Gram Staining

Few drops of deionized water were placed on glass slide and smear of bacterialcolony was formed on it. Then it was left in air to get dry. After settlement of thesmear,crystalvioletwasfloodedfor1minuteandwashedwithdeionizedwater.

Then iodine was added and washed again. Decolorizer was added for 10 seconds. At the end safranin was flooded for 40 seconds and washed and air dried. Gram positive strains were observed under microscope.

Motility of Bacterial Strains

Hanging drop method was used to examine the motility of bacterial cells underthe light microscope. Bacterial colony was added on a cover slip having water dropon it.Glass slide containing paraffin was inverted over the drop of water havingbacterialcolony.Itwasobservedunderlightmicroscope.Bacterialcellswereobserved moving around the concavity of the paraffin.

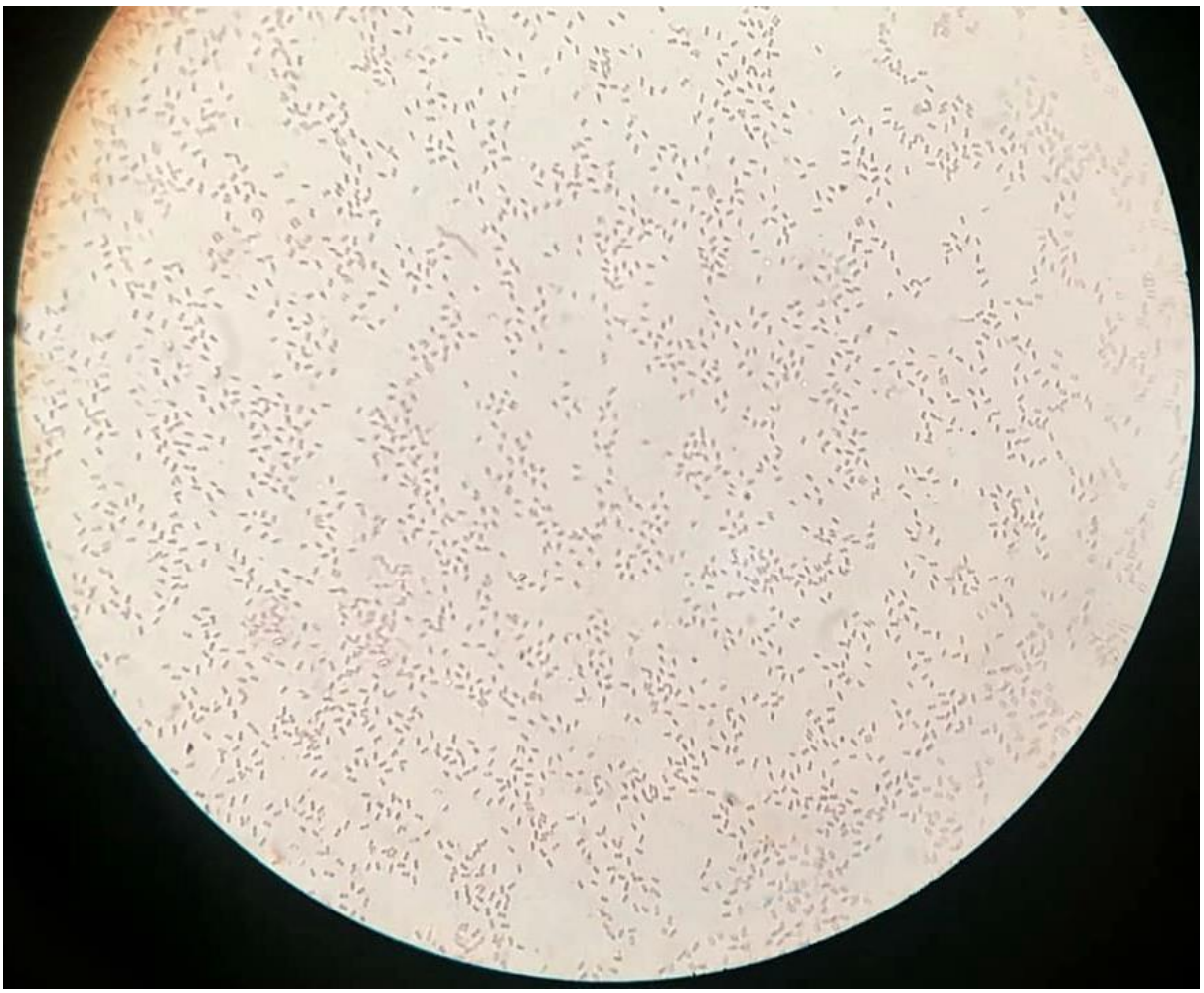

Figure 3. Gram positive strains

ENZYMEPRODUCTION

Inoculum Preparation

Bacterial isolates were grown in the liquid media containing pectin as a carbonsource,placedinincubatorat37^{o}Cfor36hours.Bacterialcolonieswereobtainedin PB after 36 hours and were used in the production media for the preparation ofpectinaseenzyme.

Fermentation

2ml of inoculums was pouredin250mlofproductionmediatoproducepectinase enzyme. It was placed in rotatory shaker with 120rpm at 37^{o}C for 48hours. After 48 hours, the fermented culture was centrifuged at 6000rpm for 10minutes. The bacterial cells were pellet down while the supernatant was taken as crude extract.

Enzyme Purification

Thepurificationprocessofenzymewasperformedusingthe$(NH_4)_2SO_4$precipitation technique following the cellulose tubing dialysis and gel filtration chromatography.

Ammonium Sulphate Precipitation

Ammonium sulphate precipitation was used to partially purify the enzymes.Differentconcentrationsof$(NH_4)_2SO_4$,rangingfrom60%-90%,weredissolvedin crude enzyme. The solution of crude enzyme and ammonium sulphate was takenin to the cellulose dialysis membrane and allowed to stir and kept for 24 hours at4^{o}C. After 24 hours the solution was centrifuged at 10000rpm for 15 minutes. The pellet was dissolved in sodium phosphate buffer and Lowry assay was applied to examin ethe concentration ofprotein. The percentage of ammonium sulphate showing the maximum activity was further used to partially purify the protein.

Table 1.Ammonium sulphate precipitation

Sample	Absorbance	Proteinconc. (mg/ml)	Activity(Units)
60%	1.984	0.455	18.51
70%	1.826	0.418	17.97
80%	1.1339	0.257	9.59
90%	1.0729	0.243	9.13

Gel Filtration Chromatography

Gel filtration chromatography was carried out using Sephadex G-100 as gel. The length of column was 20cm having diameter of 1.5cm. The length of gel bed was 12cm. 1ml of protein sample obtained after dialysis was run in the gel bed and35elutedsamplesweretakenintheeppendroftubes.Lowry'sassayandDNSassaywas carriedout to observe the total protein and activity respectively.

Table 2. Fractions of protein obtained by Column Chromatography

Fractions	OD	Concentrationof protein(µg/ml**)**	Activity(Units)
1	0.3667	79.093	3.12
2	0.4437	97	4.44
3	0.36	77.535	2.76
4	0.5208	114.930	6.25
5	0.5487	121.418	6.79
6	0.3321	71.046	2.47
7	0.3264	69.72	2.13

8	0.3662	78.976	3.32
9	0.294	62.186	1.06
10	0.5047	111.186	3.73
11	0.3422	73.395	2.99
12	0.4212	91.767	2.47
13	0.8063	181.325	9.94
14	0.8045	180.906	9.88
15	0.7807	175.372	9.27
16	0.881	198.697	11.84
17	0.883	199.162	12.03
18	0.3486	74.883	3.25
19	0.2222	45.488	3.07
20	0.4293	93.651	4.93
21	0.2552	53.162	2.87
22	0.2752	57.814	3.09
23	0.3298	70.512	3.63
24	0.2714	56.930	2.96
25	0.3664	79.023	3.93
26	0.2847	60.023	2.09
27	0.2986	63.256	2.89

28	0.2777	58.395	1.14
29	0.272	57.069	2.98
30	0.2349	48.441	2.65
31	0.2162	44.093	1.04
32	0.2291	47.095	2.29
33	0.2482	51.534	1.89
34	0.2525	52.535	1.93
35	0.2184	44.605	2.08

Maximum protein concentrations were observed in the elutions from 13 to 17.Whilemaximum activity was observed in the elutions 16 and 17.

Standard Curve

Lowry assay was performed and standard curve was made by using the varying concentrations of standard. The regression equation, y = 0.0023x + 0.012wasinsertedbyusingMicrosoftExcel.Theconcentrationofgalacturonicacid,inµg/ml,was taken as‘ y’while‘ x’was O Dat 680nm.

Lowry’s assay was performed to examine the concentrations of protein in crudeextract,ammoniumsulphatesolutionandprecipitatesofammoniumsulphatesolution dissolved in buffer. The concentration of protein in crude extract is moredue to the presence of more than one protein. The concentration of protein in crudeextract obtained was 545.335µg/ml. When the ammonium sulphate solution wascentrifuged,theproteinswerepelletdown.Theconcentrationofproteinswasdecreased when it was pellet down from ammonium sulphate solution. The pellet was dissolved in sodium phosphate buffer.

The Lowry assay of dialyzed protein was performed and graph in Fig (4) was obtained. The concentration of protein obtained was 203µg/ml. As the crudeextract was treated with ammonium sulphate followed by dialysis, which resultedin the release of small sized proteins from the cellulose dialysis tubing, therefore,proteinleftinthedialysatewaspectinaseonly,hencedecreasingtheproteinconcentration. The protein concentration for crude extract, partially purified sample and pellet protein were recorded through standard curve shown in Figure 4.

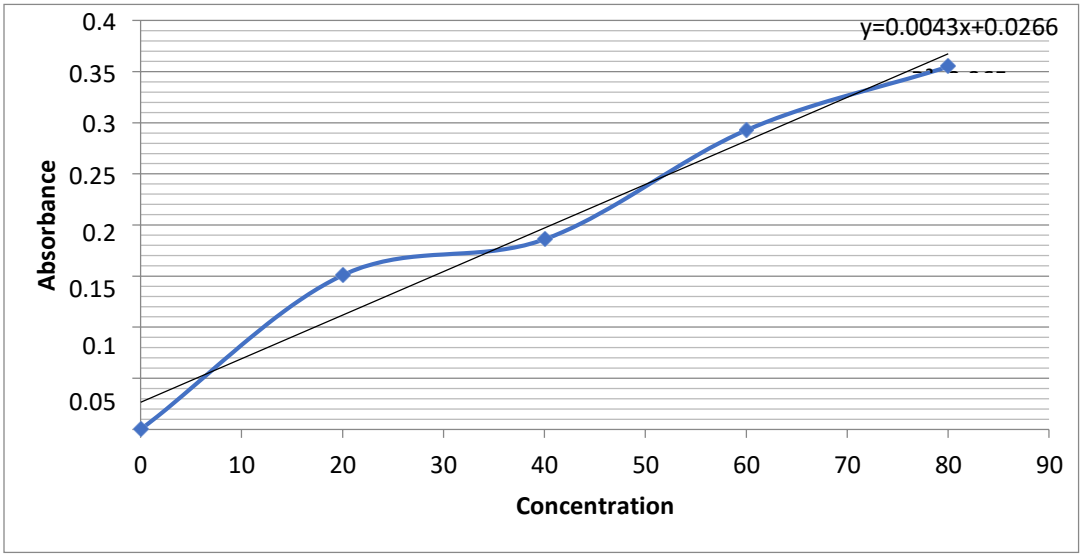

Figure 4.Standard Curve

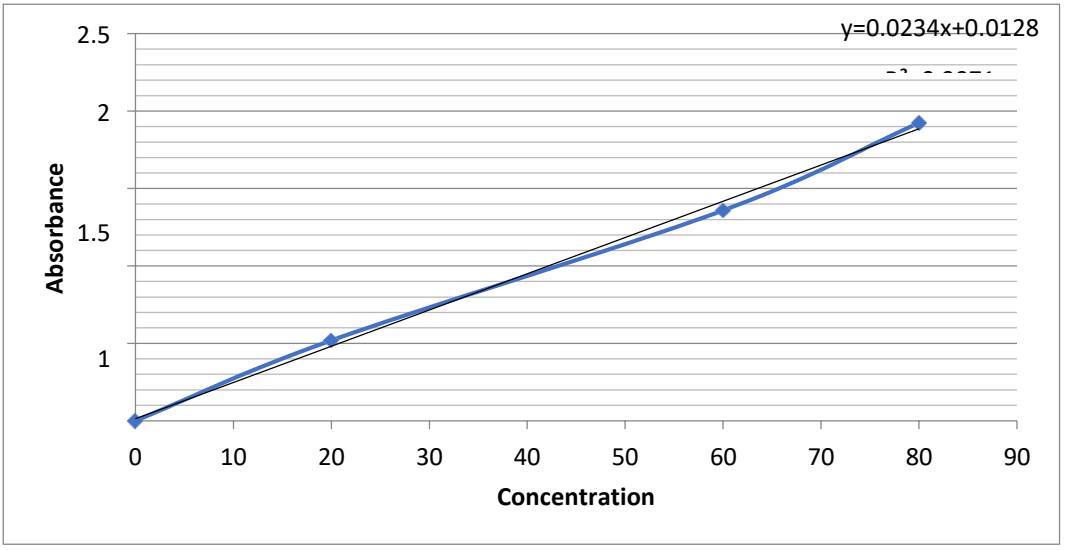

Figure 5.Standard curve

Table 3. Concentrations of sample estimated by Lowry'sAssay.

Sample	Absorbance	Conc.(µg/ml)
CrudeExtract	2.3715	545.335
Partiallypurified protein	1.588	363.162

Table 4. Purification table

Sample	Volume (ml)	TotalProtein (mg)	Activity (Units)	Specific Activity(units/mg)	Purification Fold	% Yield
Crude Extract	25	0.545	20.97	38.48	(1)	(100)
Ammonium SulphatePrecipitation (60%)	10	0.363	18.51	50.9	1.32	88
Dialysis	5	0.203	17.57	86.55	2.2	84
Column Chromatography	1	0.099	12.03	121.52	3.2	57

The activity of pectinase,12.03 units, was compared with the results of Kashyap et al. (2000) who observed maximum activity of 53 units. This difference is due to the small inoculation size and less volume of protein taken for the columnchromatography.Theconcentrationofprotein,obtainedaftergelfiltrationchromatography,observed was 0.099 mg/ml.

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EFFECT OF CLIMATE CHANGE ON AGRICULTURAL CROPS AND FOOD

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ABSTRACT

Global warming is being caused by the unchecked expansion of greenhouse gas emissions. As a result, we should expect to see changes to the seasons and a rise in precipitation and rainfall. Food security worldwide is under jeopardy as a result of climate change, rising populations, and rising incomes. The agricultural sector is particularly sensitive to global warming. In addition to promoting the growth of unwanted weeds and pests, rising temperatures ultimately lower the yields of good crops. The risk of crop failure in the near term and a loss in output in the long term is raised by changes in the distribution of precipitation. Although certain locations may see agricultural improvements due to climate change, the overall effects on agriculture are predicted to be unfavorable, posing a danger to global food security. The most at-risk populations are those living in the developing world, where poverty and hunger are already widespread. Due to these factors, food insecurity and malnutrition are increasing in rural areas, which has an impact on agricultural productivity, farm livelihoods, and agribusiness infrastructure. In order to guarantee resilient agricultural practices that ensure household food security in Pakistan, this assessment aims to emphasize the implications of climate change on the agricultural sector, present risks, and mitigation possibilities.

Key words; Climatic changes , Agriculture , Food adverse effects

INTRODUCTION

Northern Pakistan has seen a change in monsoon pattern and an increase in the incidence of cyclonesas a result of increasing temperatures, both of which have had negative effects on the

agricultural sector in recent years (Abubakar, 2020). According to a 2015 study by Ahmad et al., Pakistan has the sixth highest vulnerability to climate change globally. More people living in cities means more people affected by climate change in Pakistan (Anwar et al., 2020).

According to Awan and Yaseen (2017), Pakistan is the 12th most negatively affected nation owing to the effects of climate change on agriculture and livelihoods. Since GHG emissions from fossil fuel usage have the effect of trapping heat in the high atmosphere, they are likely to be the primary cause. The consequences of climate change were felt all over the globe as a result of this increase in global temperature, which exacerbated the global warming phenomena. Nearly 25 million Pakistanis find work in the country's agricultural sector. In addition, Pakistan is the sixth most populous nation in the world, with a growth rate of around 2% per year (Awan and Yaseen, 2017).

Pakistan is now cultivating almost all of its fertile land in an effort to meet the sustainability of food security for its fast-expanding population. The sudden shifts in rainfall patterns that have resulted from rising global temperatures have serious implications for agriculture, water supplies, and forests. Changes in the pattern and severity of rainfall have led to droughts that kill crops and floods that destroy towns and cities. Developing and implementing climate-smart crop management solutions is essential for Pakistan to attain food security, as it is for other nations that rely on arable food production in semiarid climates. Equally important is making good commitments to deploy climate change adaptation measures (Ahmad et al., 2015).

Rainfall totals, not simply their intensity, are being impacted by climate change. Increasing the nation's agricultural and irrigation infrastructure, including irrigation channels, canals, and dams for water storage, is necessary since many areas of the country get less than 250 mm of rainfall per year. It will be necessary to use the most advanced irrigation scheduling technology in

addition to implementing water saving measures. About 41% of Pakistan's greenhouse gas emissions come from livestock (Baigal, 2016). New and improved feeding technologies, altered feed compositions, improved livestock breeds, enhanced manure management practices, and pasture and grazing land management are all suggested as means to boost agricultural output and expand the soil' s capacity to store carbon (Areeja et al., 2021)

Consequences for Crops

The impact of rising temperatures on a certain crop will vary according to that crop's optimum temperature for growth and reproduction. The sorts of crops normally planted there may benefit from warming, or farmers may be able to switch to crops that are now cultivated in warmer places. However, if the greater temperature is above the optimal temperature for a crop, production will suffer.

Crop yields are vulnerable to increased CO2 levels. Evidence from the lab indicates that plants may benefit from higher CO2 concentrations. Potential improvements in production may be offset, however, by other variables like as fluctuating temperatures, ozone levels, and water and fertilizer shortages. For instance, production improvements may be mitigated or even reversed if temperatures exceed a crop's ideal level, and if enough water and nutrients are not made accessible. Alfalfa and soybean plants that have been exposed to elevated levels of CO2 have lower protein and nitrogen content. The capacity of pasture and rangeland to sustain grazing cattle may be diminished if grain and forage quality decrease(Hatfield et al., 2014).

Crops may fail to flourish if the weather becomes more volatile. Crops may be damaged and harvests might be reduced by extreme occurrences like floods and droughts. For instance, in 2010 and 2012, high overnight temperatures impacted maize yields in the U.S. maize Belt, and in

2012, $220 million in losses were incurred due to early budding of Michigan cherry as a result of a warm winter (Hatfield et al., 2014).

Rising summer temperatures may dry the soil, making drought management more difficult in certain locations. The availability of water for irrigation might be limited in areas where it is already scarce, even if greater irrigation is conceivable there.

In warmer and wetter regions with higher concentrations of carbon dioxide, many weeds, bugs, and fungus flourish. Due to weeds' competition with crops for light, water, and nutrients, farmers spend a lot of money on weed control (Rao et al., 2019). Weeds and pests may spread farther and become more widespread as a result of climate change. For farmers whose crops have never been exposed to these organisms before, this might spell disaster.

While an increase in atmospheric CO2 might spur plant growth, this comes at the expense of the food's nutritious content. Most plant species, including wheat, soybeans, and rice, have lower protein and critical mineral concentrations when atmospheric carbon dioxide levels rise. Increasing CO2 levels have a direct impact on the nutritional value of crops, which poses a risk to human health. As pest pressures rise and pesticides lose their effectiveness, there is a corresponding risk to human health from the growing usage of pesticides (Ziska et al., 2014).

CONCLUSION

Variability in climate patterns owing to increasing human activity is now seen worldwide, especially in emerging countries. South Asian nations, with their larger populations, more vulnerable locations, and less advanced technology, may be more vulnerable to the effects of a warming planet. Agricultural output would decrease as a result of the higher seasonal temperatures. Predicting the effects of climate change and developing adaptation methods may

be aided by crop growth models that include climatic and economic factors. Sustainable agricultural output might be attained via climate-smart and resilient agricultural practices by using adaptation and mitigation measures to counteract the negative impacts of climate change. To maintain agricultural yields in the face of climate change, AgMIP-Pakistan serves as a model of climate-smart agriculture. It's an interdisciplinary research strategy for determining the effects of climate change and creating site- and crop-specific adaptation technologies to safeguard food supply. Changes in crop management, such as sowing timing and density, and nitrogen and irrigation application, may be made using adaptation technologies to boost production and profitability in the face of climate change. The rice-wheat cropping system's adaptable technology may be used to produce crops sustainably in other Asian locations with comparable climates, helping to guarantee the continent's food supply. Reducing the negative impacts of climate change in Asia's most at-risk areas would need early warning systems and cross-national, trans-disciplinary research. The potential impact of climatic variability and change may be mitigated via the opportunities highlighted. Climate-resilient agricultural and animal varieties, carbon sequestration, and other climate-smart water, soil, and energy-related technologies may be promoted to increase productivity in the face of climate change. Sustainable production and food security may be aided by the use of ICT-based technologies such as EWS, AWS, decision support systems for decision-making, precision water and nutrient management technology, and crop insurance.

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EVALUATION OF SYSTEMIC FUNGICIDES AGAINST *COLLETOTRICHUM FALCATUM*CAUSING RED ROT OF SUGARCANE

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ABSTRACT

Sugarcane is an economically important crop commercially cultivated worldwide owing to huge nutritional and medicinal purposes. Annual sugarcane yield in Pakistan is rapidly minimizing due to attack of various fungal rots. To combat these threatening losses, present study was designed intensively to screen the substantial efficacy of selected systemic fungicides against red

rot of sugarcane. Morphological studies of isolated fungal rot from sugarcane crop, identified causal agent as *C.falcatum*. Two selective fungicides viz Mancozeb and Nativoalong with three different concentrations i.e., (60, 80 and 100 ppm) were evaluated *invitro*. Nativo was most effective fungicides 92.31% in inhibiting mycelial growth of *C.falcatum* following poisoned food technique. The results showed that both applied fungicides significantly affect against red rot of sugarcane, whereas no mycelial growth inhibition was recorded in control treatment. It is therefore, recommended that timely application of systemic fungicides may enhance crop yield by minimizing effect of phytopathogenic fungal rots.

Keywords; Nativo, Mancozeb, Red Rot of Sugarcane.

INTRODUCTION

Sugarcane (Saccharum officinarum L.)from family Poaceae is an important crop with global annual production recorded 531.3 million metric tons, which is almost four times higher(Shahbandeh, 2023).Grown in tropical and subtropical regions, sugarcane is the world's largest crop by in terms of production, accounting about2.1 billion tones in 2021, with Brazil accounting for about 40% of the total production. Pakistan contributes 0.9% sugarcane production share in Gross Domestic Product (GDP) (GOP, 2022).Sugarcane is one of the most significant cash crops of Pakistan contributing enormous role in the economy of Pakistan. It is grown in the tropical and subtropical regions of world. Due to its wide range of adaptability, it supplies more than 60% of world sugar and basic raw material for Pakistan sugar industry, which is second to textile industry in the country. Molasses are used for the synthesis of alcohol, bio-compost etc., while bagasse is used as fodder for farm animals during the winter season, which helps alleviating the shortage of green fodder. Cane crushed material are used as fuel in Pakistan and its juice is very popular among people of Pakistan during the hot summer season. Besides producing a vast agro-industrial base, sugarcane is an important contributor sharing (5.2%) to Gross National Product (Government of Pakistan, 2022).

Among various factors responsible for low yield, diseases are the major cause. About more than100 fungal, 20 bacterial, 12 viruses and 55different nematode species are observed pests of sugarcane around the globe (Singh and Waraitch, 1981). Red rot of sugarcane causes severe losses in sucrose yield in many cane growing areas of the world (Singh & Singh, 1989).

In Pakistan*C. Falcatum*caused serious losses in past and achieved the status of the most threatening and destructive fungal rot of sugarcane (Chaudhry et al., 1999; Subhani et al., 2008; Bharti et al., 2012).

The endless struggle between varieties and the complexity of disease have led to the development of correspondingly a variety of approaches for control. Fungicides are often a vital part of disease management as they control many diseases satisfactorily (McGrath, 2004). Red rot is a major problem for sugarcane production and is responsible for the eradication of numerous best varieties from the cultivation due to the constant evolution of the newer species (Malathi et al., 2010).Red rot pathogen hydrolyzed the stored sucrose by producing the enzyme invertage which breaks the sucrose molecule into its components namely glucose and fructose. As a result, the quantity of molasses increases and it is aptly called the cancer of sugarcane (Khan et al., 2011). As this has become major constraint in the profitable cultivation of sugarcane (Mohan and Sangeeta, 2009).

The role of fungicides in modernizing and changing the condition of agriculture is quite significant. Keeping in view the seriousness of disease, present investigations were conducted to study the efficacy of native and mancozeb on red rot of sugarcane.

MATERIAL AND METHODS

The study was designed with the aim to identify the threatening phytopathogenic rot of sugarcane crop in fields of National Agriculture research Centre Islamabad (NARC). A survey was conducted, and diseased specimens (sugarcane leaves and stalks) were brought to Fungal Plant Pathology Lab NARC. Diseased specimens were air dried for 24 hours and were pierced into 5mm pieces. After drying diseased specimens were surface sterilized with 70% ethanol and 0.5% Sodium hypochlorite solutionfor 10 minutes. Sterilized distilled water was applied for washing the specimen and were again surface sterilized with chlorax. Small 2mm portion of diseased specimen was isolated on potato dextrose agar and petri plates were incubated at 27° C for one week in incubator. After week ofincubationgrowing edges of fungal hyphae developing from tissues were transferred to PDA. Pure cultures were preserved in 15% glycerol solution and were stored at -20° C. Morphological and microscopic studies were conducted to exactly identify

the pathogen. Moreover, Pathogenicity assay was conducted to confirm Koch's postulates.Isolated fungi were inoculated into6-7weeks old sugarcane plant grown in greenhouse. Fungal rot wasisolated on PDA petri dishes for 7 days 27°C in incubator and was further re-inoculated firstly, by conidial suspensionsprayed on the insertion of leaves and secondlyby inserting two mycelial plugs of 5mm diameter taken from the fungal colonies into the sugarcane stalk. The inoculated stalks were placed in the trays and kept in laboratory at room temperature.Diseased symptoms were observed daily after post inoculation and were compared to fungal taxonomic keys for exact disease identification. After 9 days stalks were cut down longitudinally to observe internal symptoms.

Invitro*Managementof *Colletotrichum falcatum

Poisoned food technique assay was conducted to screen efficacy of applied fungicides against isolated fungal pathogen. Percentage MycelialInhibition was recorded after all odd DPI (Days Post Inoculation) for one week.Fungicides (Nativo and Mancozeb) were applied at three concentrations (60, 80 and 100 ppm) whereas, sterile distilled water was applied as control treatment. All the three concentrations of selected fungicides were individually added into PDAand media was poured into 5 Petri plates of 90mm diameter. Once media gets solidified into petri plates, a small disc of 7 days old pure culture of isolated fungi wasand placedin centre of at equilibriuminto each poisoned media plates.Petri dishes were incubated for a week in incubator at 25 C. Readings were noted after all odd DPI's. Mycelial Percentage inhibition of fungus was calculated by following formula.

$$I\ (\%) = (C\text{-}T/C) \times 100.$$

RESULTS AND DISCUSSION

Morphological and microscopic studies identified the fungal pathogen as*Colletotrichum falcatum*. Among a total of 10 isolates, 4 were observed 89white, gray-colored colonies with a dark and gray conidial mass in the center. The results revealed that the isolates of *Colletotrichum falcatum* produced hyaline cylindrical conidia. 3 isolates were recorded having creamish-white colonies with a salmon-gray colored conidial mass exactly at the center and fusiform tapered to a point in both ends. Remaining 3 isolates were observed showing pure creamish to light orange

mycelium with reverse side of plate showing blackish to light, gray-colored conidial mass at the center with cylindrical conidia having obtuse to slightly rounded ends. The length of conidia ranged from 10.7-15.5 µm(Table 1).

Table 1. Morphological Characteristics of *Colletotrichum falcatum*

S. No	ISOLATES	Conidia Shape	Appressoria Shape	Size	Color	Pathogenic
1	CFM1	Falcate	Ovoid-irregular	10.1-11.1 µm	Whitish to Gray color	Moderately Pathogenic
2	CFM2	Sickle Shaped	Ovoid-irregular	10.5-13.4 µm	Whitish to Gray color	Severely Pathogenic
3	CFM3	Sickle Shaped	Ovoid-irregular	10.1-11.4 µm	Whitish to Gray color	Highly pathogenic
4	CFM4	Sickle Shaped	Ovoid-irregular	10.6-15.4 µm	Gray-colored	Highly pathogenic
5	CFM5	Sickle Shaped	Ovoid-irregular	8.4-13.5 µm	Creamish-light orange	Severely Pathogenic
6	CFM6	Falcate	Ovoid-irregular	9.4-14.1 µm	Creamish-white	Severely Pathogenic
7	CFM7	Falcate	Ovoid-irregular	10.2-11.4 µm	Creamish-white	Highly pathogenic
8	CFM8	Falcate	Ovoid-irregular	10.2-12.4 µm	Creamish-light orange	Severely Pathogenic
9	CFM9	Falcate	Ovoid-irregular	10.5-11.6 µm	Creamish-white	Moderately pathogenic
10	CFM10	Falcate	Ovoid-irregular	10.5-15.4 µm	Creamish-light orange	Highly pathogenic

EFFICAY OF FUNGICIDES AGAINST *C. FALCATUM*

Fungicides are specific in their action and specificity for various genus and species. Analysis of variance showed significant difference in the effectiveness of both tested fungicides, their doses and the interaction between doses and fungicides for the suppression of radial growth of *C. falcatum*. Results of poisoned food technique showed that maximum inhibition of *C. falcatum* was observed by Nativo viz; 94.51%, 96.22% and 96.91% at all applied concentrations (60, 80 and 100 ppm) after one week of incubation. Whereas application of Mancozeb at all applied concentrations also significantly inhibited fungal mycelium viz; 89.45&, 93.91% and 94.72%, respectively (Figure 1 and 2).

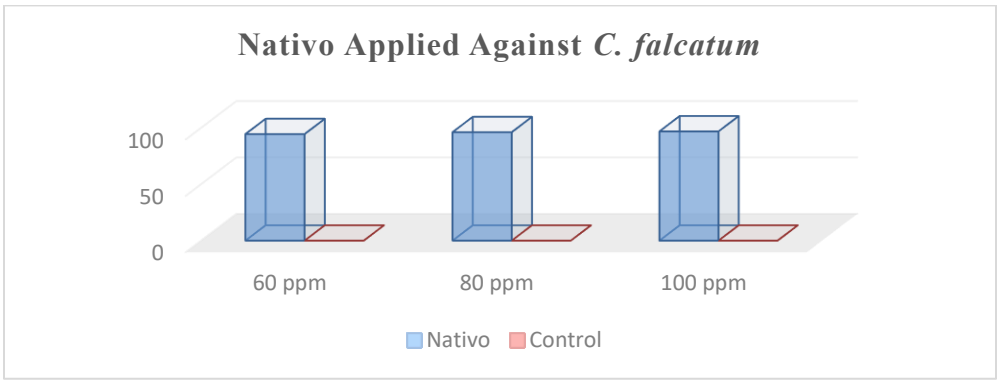

Figure 1.Nativo applied at three concentrations (60 ppm, 80 ppm, 100 ppm) Control* No mycelial inhibition observed

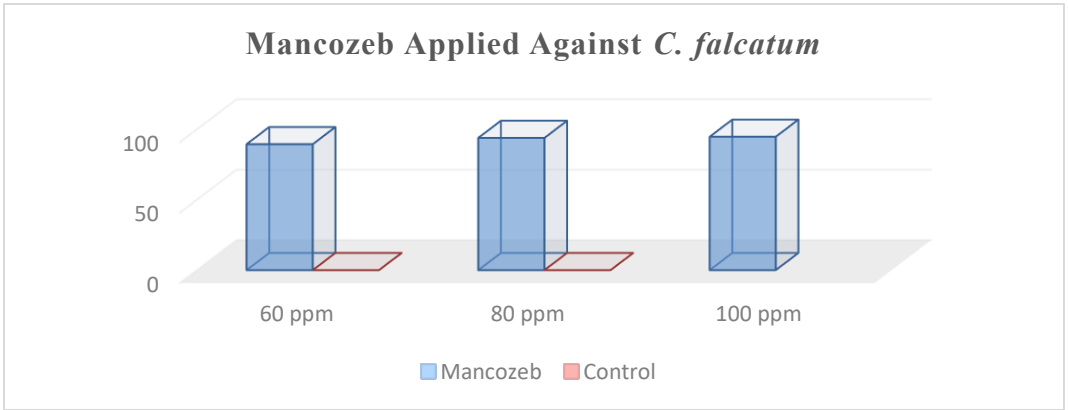

Figure 2. Mancozeb applied at three concentrations (60 ppm, 80 ppm, 100 ppm) Control* No mycelial inhibition observed.

Cultivation of resistant varieties is the most economical and efficient technique adopted worldwide to manage red rot of sugarcane (Viswanathan et al., 2009) but when disease free seed is not in approach of farmers than no other option is left except application of systemic fungicides. Chemical control is an easy-going and rapid method against various threatening fungal diseases of cereal crops. In the present study *invitro* screening of fungicides against red rot of sugarcane was evaluated where maximum inhibition of *C. falcatum* was observed by Nativo viz; 94.51%, 96.22% and 96.91% at all applied concentrations (60, 80 and 100 ppm) after one week of incubation. Whereas application of Mancozeb at all applied concentrations also significantly inhibited fungal mycelium viz; 89.45,93.91% and 94.72%, respectively. Moreover, other researchers also reported efficacy of bio-fungicides which produce gliotoxin and viridin and can certainly inhibit the growth of *C. falcatum* (Singh et al., 2004; Kumar, 2019).

Another study conducted by Subhani et al. (2008) revealed that among Twelve different fungicides screened against red rot of sugarcane, the best effective were Benomyl 50 WP, Folicar and Radomil 75 WP, which completely inhibited the growth of fungus at 5, 10, 20 and 50 µg mL^{-1}.Similarly, in another study it was observed that thiophanate methyl was highly effective against red rot of sugarcane at 10ppm (Malathi et al., 2013).

CONCLUSION

Present research summarizes that Nativo is the best effective systemic fungicide against red rot of sugarcane initiated by *C. falcatum*. All the three applied concentrations of nativoandmancozeb significantly suppresses fungal mycelium at all odd DPI (Days Post Inoculation). The growth and proliferation of red rot pathogen is affected by various environmental and nutritional conditions also, therefore timely applications of particular dose of systemic fungicides may play an excellent inhibitory role against fungus and promote enhanced yield.

CONFLICT OF INTEREST

The authors declared no potential conflict of interest.

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EVALUATING EFFICACY OF *TRICHODERMA HARZIANUM, T. VIRIDE* AND *T. ASPERELLUM*AGAINST DRY ROT OF POTATO IN PAKISTAN

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ABSTRACT

Fusarium sambucinum is one of the most aggressive fusarium specie initiating potato dry rot diseasearound the world and causes significant economic losses. To alleviate reliance on synthetic fungicides as their long-term use may lead to fungal resistance, biocontrol assays against threatening fungal diseases hold significant importance as an eco-friendly management approach.Antagonists are gradually becoming the hotspot and hold broad applicable prospects. *Trichoderma harzianum,T. viride* and *T. asperellum*were screened for their biocontrolaction against highly pathogenic isolates of*F. sambucinum* causing dry rot of potato in Pakistan. *In vitro* dual culture assay, observed after incubation at 27°C for one week, showed that all the three tested *Trichoderma* species considerably inhibited the radial growth of*F. sambucinum* as compared to the control treatment where no visible fungal mycelial inhibition was recorded. Our results revealed that using antagonists against globally threatening fungal diseases may enhance quality and production of important cash crops by generating handsome revenue.

Keywords; *Fusarium sambucinum, Trichoderma harzianum,T. viride* and *T. asperellum*

INTRODUCTION

Dry rot of potato is a devastating fungal disease of potato (Solanum tuberosum L.) affecting quality and quantity of crop. It is observed that dry rot of potato declines annual worldwide production of potatoes to about 57%. Whereas the yield losses recorded in field is approximately 23% (Stevenson et al., 2001). Potato is a first non-cereal food crop for human consumption and holds high potential in food security in developing nations (Tiwari et al., 2019). About 1.3 billion people consume fresh potatoes as a staple food (50 kg per person annually) in India and China.

InPakistan, potatois cultivatedin various areas. In Punjab, about 83 percent of the potato production originates followed by Khyber Pakhtunkhwa 10%, in Baluchistan6% and in Sindh 1 % potato are cultivated(Khalil et al., 2021).Potatoes are grown over a large area of Pakistan with the production of roughly 4.1 million tons. They are largely grown in the central and northern plains of Punjab and KPK. Some parts or districts of Baluchistan in west supporting the production of potato, include Pishin, Killa Saifulla, Kalat and Gilgit district in Gilgit-Baltistan. Potato in Pakistan is not yet being produced to its maximum capacity as compared to neighboring India and Bangladesh (Majeed and Muhammad, 2018).

Primary causes of potato dry rot disease are several species of Fusarium such as *F. sambucinum, F. solani, F. avenaceum, F. culmorum* and *F. oxysporum* (Stefańczyk et al., 2016). Moreover, Potato crop is attacked by various other plant pathogens among which fungal phytopathogenic rots are major destructive concerns declining potato production worldwide. Major fungal diseases of potato are early blight, late blight, black scurf, Fusarium wilt(Aydin et al., 2016). The most important dry rot pathogen is *Fusarium sambucinum* (Tiwari et al., 2019). This Fungus can survive as chlamydospores for many years in soil and plant debris. The temperature ranges 59°F and it require high humidity. The pathogen enters through wounds or natural opening. The tubers and roots are the main plant parts that are directly affected by dry rot disease(Secor and Sales, 2001).The economically significant part of the potato is tuber, site for storage of carbohydrates and consists of about 77% water, 20% indigestible carbohydrate (Zaheer and Akhtar, 2016).

The disease can temporarily be suppressed by some postharvest applications to the tubers. However, when tubers exposed to the pathogen in soil, no measures can be taken except application of Trichoderma as a antagonist (Gachango et al., 2012). The different efficacy of the biocontrol agents could be due to the influence of several factors viz; efficiency of the type or strain of biocontrol agent, the type of pathogens, the susceptibility of the host to the pathogen and environment (Elad et al., 1980).It is pertinent to mention that effective tuber treatments along with other applications prior to planting or during storage may reduce the severity of dry rot disease (Daami-Remadi et al., 2006. Therefore, the main objective of the proposed research was to screen and evaluate the bio-efficacy of different *Trichoderma spp.,*against dry rot of potato.

MATERIAL AND METHODS

The experiment was conducted at National Agriculture Research Centre (NARC) Islamabad with the objective to combat efficacy of biocontrol agents against dry rot of potato. Potato tuberswith visiblesymptoms of dry rotwere collected from potato fields at NARC and werecarriedin polythene bags toFungal Plant pathology Lab. Samples were air-dried for 24 hours, surface sterilized with 70% ethanol and were pierced into 5mm pieces. The pieces were cultured on PDA mediaand incubated at 25°C in the light for 7 days. The conidia were isolated using the single

spore isolation technique. Morphological and microscopic features were also measured according to protocol of (Aydin et al., 2018).

Furthermore, the pathogenicity assay was conducted to determinethe pathogenic nature of isolated fungal.Two mycelial plugs (5mm) diameter were taken from the pure culture of fungal colonies grown on PDA media. Potato tubers were surface sterilized with 70% ethanol and the mycelium plug was inserted into the tubers.The inoculated tubers were placed in the trays and kept in laboratory at25°C. Diseases symptoms were observedafter all odd DPIand werecompared with purifieddry rot disease culture on PDA.After 9 days, potato tubers were cut down longitudinally to observe internal symptoms. Koch Postulates were confirmed according to protocol of Marileideetal.(2021).Moreover, fungal isolates were preserved in slants for long termusage.

Dual Culture Assay

Three species of Trichoderma viz; *Trichoderma viride, Trichodermaharzianum* and*Trichodermaasperellum* were cultured on PDA media and incubated for 7 days at 25°C in incubator. Dual culture assay technique was conducted for the management of *F. sambucinum* by placing a 5mm diameter mycelial discs of 7-day old culture of *T. viride, T. harzianum* and *T. asperellum* individually along *F.sambucinum* petri plates. All experiments were repeated in triplicate along with three replications (FSRM11, FSRM18, FSRM29) isolates at eqi-distance from the periphery. Plates were incubated for 7 days at 25°C. Petri plate without antagonists were used as a control treatment. The mean radial mycelial growth was equally measured after all odd DPI.

RESULTS AND DISCUSSION

Fungal colonies were observedwithwhite velvety appearance above and pinkish-white color undernear the surface of the petri plates (Figure 1a and b).Colonies were observed with rapid growth of about 4.5cm in four days with arial mycelium having white to cream color. Thick wall vegetative cell chlamydospores were observed with oval to ellipsoid septate hyaline hyphae that was measured 3-7 diameter in microns (Figure 2 a; Table 1). Moreover, four-seven septations were observed in macro-spores. Macroconidia were formed after 4 -7 days from short multiple branched conidiophores. Based on morphological features the fungal rot was identified as

*Fusariumsambucinum*initiating dry rot of potato.Pathogenicity assay was conducted where three isolates of *F. sambucinum* viz; FSRM11, FSRM18, FSRM29were revealed as highly pathogenic in nature and were further selected for management experiment.

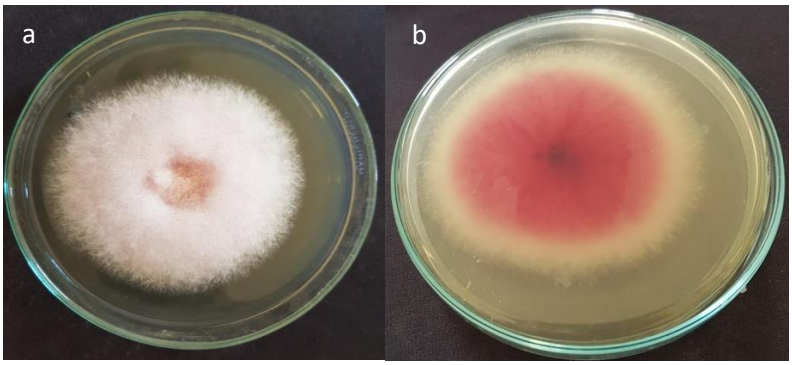

Figure 1 (a and b) Colony appearance of *F. sambucinum* on PDA, Chlamydospres

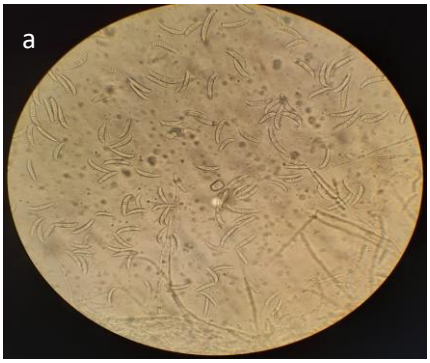

Figure 2 (a) Chlamydospores of *F. sambucinum* observed under Nikon micoscope

Table 1.Morphologicalfeatures of *Fusarium sambucinum*

S.No	FSR	Spores	Shape	Size	Color	Septation	Hyphae	Pathogenic
1.	FSRM11	Chlamydospores	Oval to ellipsoid	3-7 diameter	Whitish to pink color	4-6 septation	Hyaline	Moderately
2.	FSRM18	Chlamydospores	Oval to ellipsoid	3- 7 diameter	Whitish to pink color	5-7 septation	Hyaline	Severely
3.	FSRM29	Chlamydospores	Oval to	4- 6	Whitish to pink	4-7	Hyaline	Highly

	ellipsoid	diameter	color	Septation	pathogenic

Dual Culture Assay

All the three*Trichoderma* species act as an excellent antagonist against *F. sambucinum* isolates (FSRM11, FSRM18, FSRM29) whencross planted in dotted pattern on the PDA medium. Furthermore, it was observed that *T. harzianum* inhibited mycelial growth of *F. sambucinum* isolatesviz; 91.61%, 93.20% and 94.1% followed by *T. viride*inhibiting radial growth of fungus 86.22%, 86.9%, 93.91% and *T. asperellum* as an antagonist was recorded suppressing fungal mycelium viz; 83.91%, 93.22% and 85.91% respectively (Figure 2). Whereas no visible growth inhibition was recorded on control treatment. The most efficient antagonists for *F. sambucinum*FSRM29 were*T. harzianum* and *T. viride*. Theantagonists for *F. sambucinum*FSRM18 were*T.viride* and*T.asperellum*whereas of *F. sambucinum* FSRM11 were *T. harzianum* and *T. viride* respectively. Trichoderma isolates showed hyper parasitic nature and inhibited the mycelial growth of fungal colonies by growing over them completely by showing strong resistance.

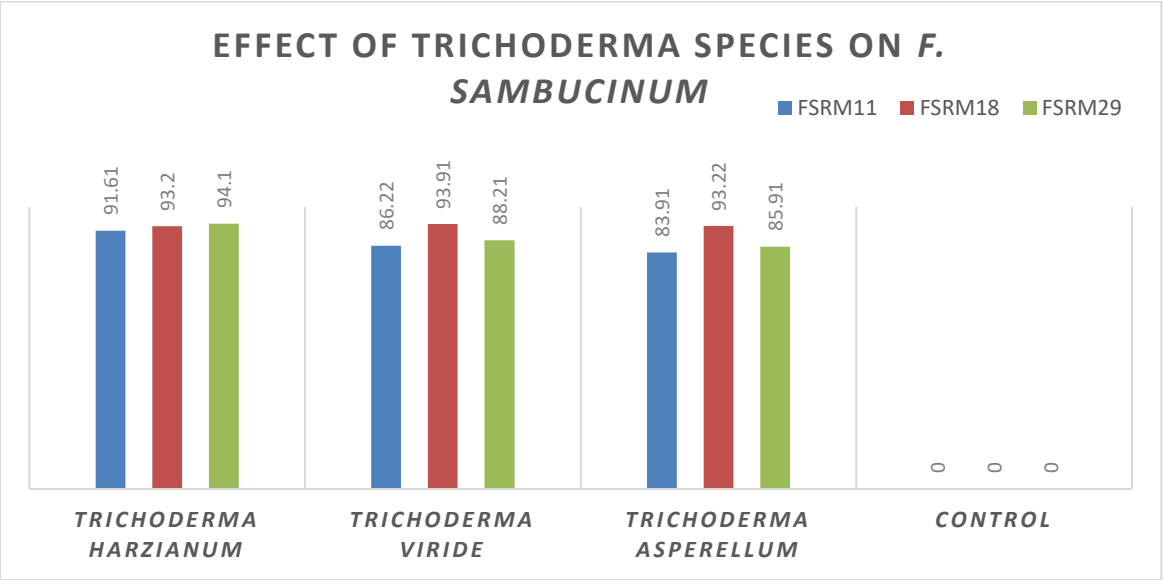

Figure 2. Effect of Trichoderma species on *F. Sambucinum*

Our study emphasizes an important role in investigating the use of biological control agents as bio pesticides and bio herbicides against phyto-pathogenic fungi by improvingand promoting crop yield. In the present study the most efficient antagonists of Trichoderma against *F. sambucinum*isolate FSRM29 were*T. harzianum* and *T. viride*. The antagonists for *F. sambucinum*isolate FSRM18 were *T. viride* and *T.asperellum*whereas of *F. sambucinum* FSRM11 were *T. harzianum* and *T. viride* respectively. Trichoderma isolates showed hyper parasitic nature and inhibited the mycelial growth of fungal colonies by growing over them completely by showing strong resistance.

The results showed that some Trichoderma species reduced the dry rot disease that occurs in the tubers. The results of our study are in good agreement with some of previous studies whereDaami-Remadi et al. (2006) reported that *T. harzianum* and *T. viride*inhibited mycelial growth of *F. sambucinum* effectively.Various Trichoderma species viz; *T. atrovirde, T. longibrachiaum, T. virens* and *T. hazianum* act efficiently against *F. sambucinum* where *T.virens* and *T.hazianum*were highly effective (Gonzales et al., 2002). There are a lot of studies in the literature indicating that Trichoderma sp. may be used as potential biocontrol agents against potato dry rot disease (Schisler et al., 1998; Dolničar, 2021). In contrast to the earlier studies, multiple isolates which were different from each other in terms of virulence were used in this study whereTrichoderma species efficiently verified the competence on all pathogenic isolates of *F. sambucinum*.

Moreover, different*invitro* and *in vivo*studies support the research that antagonism is the main mechanism for biological control of disease(Peter et al., 2008).

The use of fungal biocontrol agents to control potato dry rot disease holdssignificant eco-friendly importance as post-harvest conditions are an ideal point for efficacy of biocontrol agents (Ru and Di, 2012).In this present study, applied antagonistsshowedgreat inhibition activity against potato dry disease.

CONCLUSION

Potato dry rot initiated by *F. sambucinum* is a devastating field and a postharvest fungal rot decling quality and quantity of potato crop globally. To mitigate reliance on synthetic fungicides antagonism by biocontrol agents holds significant attraction among scientists. Application of

Trichoderma harzianum, T. viride and *T. asperellum* suppressed the mycelial growth of potato dry rot fungus in the present study and emphasizes future use of these biological agents as an eco-friendly, economical and natural biocides.

Conflict of Interest

The authors declare no potential conflict of interest.

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SCREENING OF SYSTEMIC FUNGICIDES AGAINST *FUSARIUM VERTICILLOIDE* INITIATING STALK ROT OF MAIZE

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ABSTRACT

Maize is an important cereal crop with huge global attraction and significant role as human food and animal feed. Various phytopathogenic fungal rots infect quality and quantity of maize before and after harvesting. The present study was designed intensively to combat the major objective of screening systemic fungicides against *Fusarium verticilloide* initiating stalk rot of maize. Various concentrations (20 ppm, 40 ppm) of synthetic fungicides *viz,* Dithiocarbamate, Ebuconazole 50% + Trifloxystrobin 25% and Prochloraz were evaluated in-vitro against *F. verticilloide.* It was revealed that Tebuconazole 50% + Trifloxystrobin 25%at 60 ppm showed the strongest anti-mycotic potential83.6%followed by Prochlorazshowing 75.19%mycelial inhibition of fungal rot, whereas dithiocarbamate was least effective at 7th day post inoculation respectively. Summarization of our results signified that trifloxistrobin 25% + tebuconazole 50% precisely controlled the maximum radial growth of *F. verticilloide*. It is therefore suggested that

timely use of these fungicides may help in enhancing overall maize yield with good economic returns.

Keywords; *Fusarium verticilloide*, Dithiocarbamate, Tebuconazole, Trifloxystrobin, Prochloraz.

INTRODUCTION

Maize (Zea mays L). from family Gramineae, is a globally important cereal crop that ranks third after rice and wheat accordingto area and production. Maize was first domesticated for about 9,000 years ago in southern Mexico/Meso America (Karlsson et al. 2021).The global maize area (for dry grain) accounts 197 M ha, including substantive areas in sub-Saharan Africa (SSA), Asia and Latin America (FAO, 2021). The global maize area is primarily located in the America and Asia, with over a third each, followed by Africa with a fifth and Europe with a tenth. Maize is also called queen of cereals due to high yield population among all cereals (Kenganal et al. 2017).

In Pakistan, the average productivity of maize is 2,850 kg/ha, which is the highest among all cereals grown in the country. The bulk (99%) of total production comes from two major provinces, NWFPaccounting for 52% of the total area and Punjab contributing 48% 9Hussain et al. 2022). It is pertinent to mention that maize isalso an important cereal crop of Azad State of Jammu Kashmir Pakistan withabout 0.122 million ha cultivated in autumn (GOAJK, 2022). It is an established and important human food crop in a number of countries, especially in SSA, Latin America, and a few countries in Asia, where maize consumed as human food contributes over 20% of food calories (Shiferaw et al., 2011*)*. The share of maize in grain production is 1.8 million tons per annum (Ahmad et al. 2017).US is the largest producer of maizecrop, and it dominates world maize trade (Ranum et al. 2014).

Major fungal diseases affecting quality and quantity of maize crop includefusarium rot, root rot, stalk rot, seedling blight, head blight, charcoal rot, etc. Fusarium is one of the major devastating groups of plant-pathogenic fungi affecting a huge diversity of crops in all climatic zones across the globe.Various fusarium species viz; *F. verticillioides*, F. *graminiacum, F. sporotrichioides, F. sambucinum,* etcinfect maize grains after harvesting in storage and during transportation. Maize is particularly susceptible to *F. verticillioides* infection due to the large amounts of

fumonisins produced, whereas *F. verticilliodes* is a toxigenic fungus with ability to survive under harsh weather and high temperature (Czembor et al., 2015).

Among which major rots observed and reported are*F.proliferatum, F. graminearum* and *F. anthophilium* (Abake et al. 2015).Fusarium spp. produces *gibberella* ear rot, kernal rot, seedling blight, seedling rot, wilt and smut respectively and are considered the largest group of seed born fungal rots (Kumar et al., 2013). Fusarium species invade 50% of maize grain before harvest and it produces mycotoxins(Bacon, 2001). Fungi is the second most cause of deterioration and loss of maize (). Fusarium species produces mycotoxins such as monilliformin, fumonisin and fusaric acid (Cotton and Munkvold, 1998).A high incidence of stalk rot has been reported in several maize fields all over the world. Maize stalk rot is associated with *Fusarium temperatum, Fusarium subglutinas* and *Fusarium verticilloide*. With the introduction of high yielding hybrids both indigenous, exotic and use of fertilizers, there is a phenomenal increase in the area and production, but at the same time, it is prone to several foliar and stalk rot diseases (Payak and Sharma, 1980). Fusarium rot can infect any part of maize straight starting from beginning to end of growing season. Stalk rot is the major and severe fungal rot on maize causing reduced growth, rotted leaf sheaths, internal stalk tissue and brown streaks in lower internodes. It causes pink to salmon discoloration of the internal stalk pith tissues (Munkvold et al., 2021). Fusarium stalk rot occurs in warm and dry region and produces black perithecia (sexual fruiting bodies) in infected maize stalk. The infection results in premature death of maize plant as pathogen interferes with the translocation of water and nutrients (Geiser et al., 2013).

This study was aimed to determine the most prevailing pathogen of stalk rot of maize crop field located at National Agriculture Research Centre Islamabad and to screen the efficacy of various fungicides against Stalk rot of maize. Different fungicides were evaluated *in vitro* against stalk rot diseasewhere the most effective synthetic fungicide was further suggested for future use in enhancing overall maize yield production with good economic returns.

MATERIAL AND METHODS

The research was conducted at National Agriculture Research Centre Islamabad, Pakistan.Maize stalks with clearly visible rot symptoms were brought to the Fungal Plant Pathology laboratory in polythene

bags from maize fields and were air-dried for 24 h. Later on, stalks were pierced into 5-mm pieces and were surface sterilized with 0.5% Sodium Hypochlorite solution for 3 minutes and were washed with 70% Ethanol for 2 minutes followed by three washings with sterile distilled water. The pieces were placed on potato dextrose agar separately and were incubated at 25°C for 72 hours. Morpho-molecular studies were conducted under 40X Nikon Microscope.

Pathogenicity assay was conducted by harvesting conidia of *F. verticilloide* by scraping the surface of 3-day-old culture plate flooded with sterile distilled water (SDW) using the bottom of a 1.5-ml micro centrifuge tube. Mycelial fragments were removed by filtration through two layers of Miracloth. The conidia were pelleted by centrifugation, washed and diluted to approximately 4×106 conidia/ml in distilled water. A total of 20 seeds of maize were planted in pots containing sterilized soil and placed in a greenhouse. A conidial suspension (1 ml/ was injected into stalks of the 3-week-old maize plant using toothpick method. Isolated fungal rot was later preserved in 15% glycerol solution and stored at -20°C.

In-vitro* Evaluation of Fungicides against *Fusarium verticilloide

To screen the inhibition percentage of *Fusarium verticilloide*inoculum Poisoned food technique was followed by using different fungicides along with various concentrations. PDA was prepared and autoclaved at 121°C for 20 min. Different concentrations of Dithiocarbamate (20 ppm, 40 ppm)Tebuconazole 50% + Trifloxystrobin 25%(20 ppm, 40 ppm)and Prochloraz (20 ppm, 40 ppm)were added to the PDA media made in a volume of 100 ml.PDA waspoured into 5 Petri plates (90mm) of each concentration and were solidified.A small disc of 7 days old culture of *Fusarium verticilloide*was placed perpendicularly at the center of each petri dish and were incubated at 25°C for 7 days. Readings were taken after 5 and 7 odd day interval. Percentage Inhibition of radial growth of *Fusarium verticilloide*was calculated according to formula.

I(%) = (C-T/C) ×100

Data Analysis

Data Analysis

RESULTS AND DISCUSSION

Morphological features of isolated fungi were observed with yellowish-white colonies on PDA and the average size of micro conidia ranged from 4.0-33.0 µm x 2.4-3.3 µm.The shape varied from oval-club with flattened base havingno septations. The micro-conidia were abundant in aerial mycelia, formed long chains with false head attached at Monophialides branches.The average size of macro conidia ranged from 37.0-55.0 µm x 4.0-4.2 µm with curved-tapered shape showing a pointed notched or foot shape showing 3-5 septations. The fungus produces a copious amount of single-celled micro-conidia and plentiful amount of septate macro-conidia. In another study of microscopic features of *Fusarium* chains of micro-conidia were present whereas chlamydo spores were totally absent.

Colonies are white to pale salmon colored, with low and often ropy mycelium and a powdery texture due to production of micro conidia.

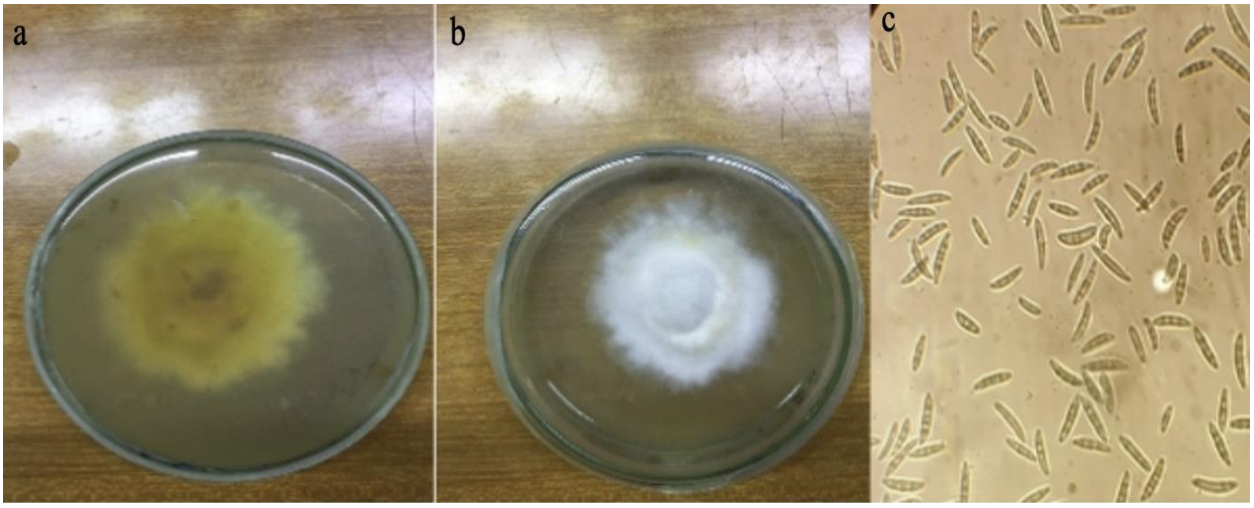

Figure 1. Pure culture of *Fusarium verticilloide* (a and b) lower and upper surface of culture on petri-dish, (c) Spores observed under 40X Nikon Microscope Magnification

In-vitro Evaluation of Fungicides against *Fusarium verticilloide*

Results of in vitro evaluation of systemic fungicides revealed that all the tested three systemic fungicides exhibited significant effect on radial mycelial growth of *Fusarium verticilloide* where the radial growth was decreased with increase in concentrationsof test

fungicides. Similarly, in another study reported by Cotton and Munkvold in 1998, *Fusariumsubglutinans*, *Fusariumtemperatum* and *Fusarium graminearum*were reported as major pathogens causing stalk rot on maize in various countries.

In the present study stalk rot of maize was observed associated with *Fusarium verticilloide*.Fusarium stalk rot occurs in warm and dry region and produces black perithecia (sexual fruiting bodies) in infected maize stalk. The infection results in premature death of maize plant as pathogen interferes with the translocation of water and nutrients (Geiser et al. 2013).To investigate the effects of different fungicides on *F.verticilloide* three selective systemic fungicides were applied.

In another similar study, significant efforts in the development and use of fungicides against radial growth inhibition of *Fusarium verticillioides* and to maximize yield in cereals several reports focus on evaluation of synthetic fungicides (Tolylfluanid M, Pencycuron, Captan, Thiram, Thiabendazole, Iprodione, Carboxin, Thiram) where it was observed that thiabendazole was the most effective in *F. verticillioides* control (Afolabi et al., 2008).

In present study three fungicides (Dithiocarbamate, Tebuconazole 50% + Trifloxystrobin 25% and Prochloraz) were tested against *Fusarium verticilloide* under in-vitro conditions through poison food technique. The data regarding the percent growth inhibition by these fungicides proved that Tebuconazole 50% + Trifloxystrobin 25%is the most toxic fungicide against *F. verticilloide* which inhibit 69.1% in C1 followed by C2 72.7% at 5thDPI, whereas C1 and C2 inhibited mycelial growth of phytopathogenic fungi on 7th DPI75.11% and 83.6% respectively (Table 1).

Table 1.Measurement of Mycelial Inhibition of *F. verticilloide*after 5th and 7thDPI (Days Post Inoculation)

Sr. #		**5th DPI (%)**		**7th DPI (%)**	
	Fungicides	**Conc.1**	**Conc.2**	**Conc.1**	**Conc.2**
1	Dithiocarbamate	67.21	69.11	69.43	71.2
2	Tebuconazole 50% + Trifloxystrobin 25%	69.1	72.7	75.11	83.6

3	Prochloraz	69.0	69.47	70.1	75.19
4	Control	0.00	0.00	0.00	0.00

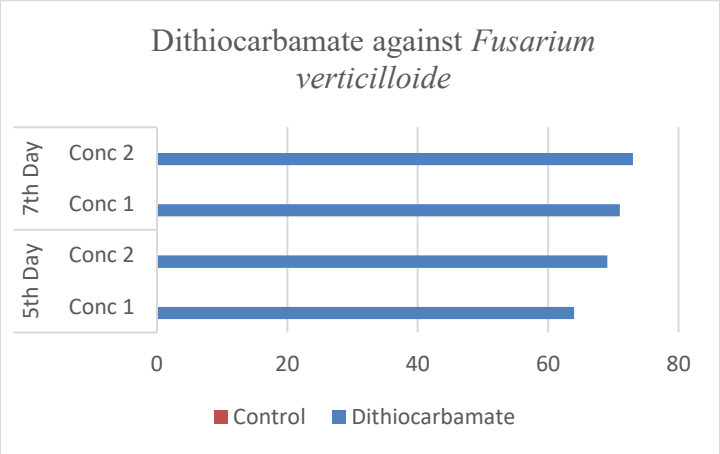

Figure 2)Mycelial inhibition of *F. verticilloide* by C1 after 5th day, C2 after 5th day, C1 after 7th day, C2 after 7th day. Control* No visible mycelial inhibition recorded.

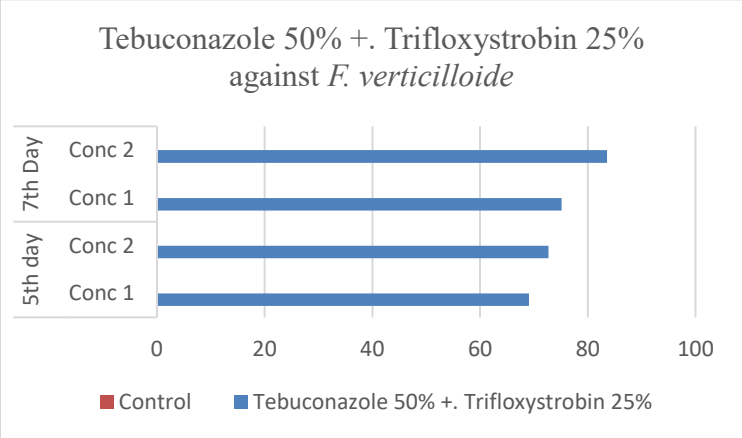

Figure 3. Mycelial inhibition of *F. verticilloide* by C1 after 5th day, C2 after 5th day, C1 after 7th day, C2 after 7th day. Control* No visible mycelial inhibition recorded.

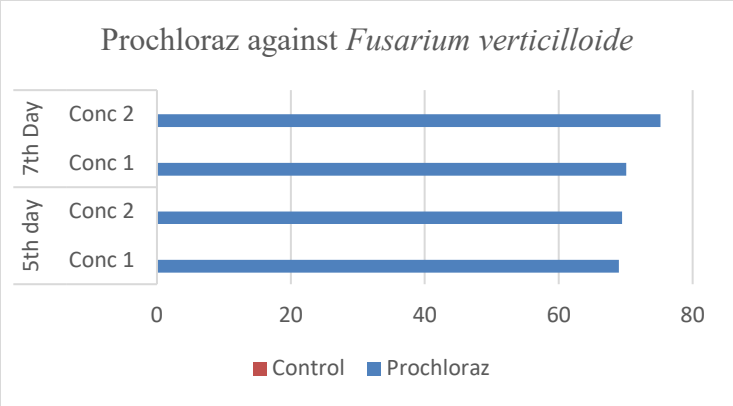

Figure 4. Mycelial inhibition of *F. verticilloide* by C1 after 5th day, C2 after 5th day, C1 after 7th day, C2 after 7th day. Control* No visible mycelial inhibition recorded.

Maize crop contributes toward economy and food security around the globe. Maize fungal diseases preferably caused by Fusarium spp., are not specifically immense to the plant but to the entire biotic community also. In various studies many fungicides were tested against stalk rot of maize by other researchers and found many of them were extremely effective (Musmade et al. 2013). Fungicides have played a major role in the management of devastating crop diseases and thereby realizing avoidable yield loss. It is known fact that their application is unavoidable in control of more dreaded plant pathogens against which host resistance is not easily available or is unstable, especially against the polycyclic pathogens. Further, most of the crops cannot remain disease free during the cropping season and hence greatly dependent on the use of fungicides. At present more than 200 chemicals of diverse classes are in use in the world as fungicides (Ajayi, 2020). Hence, it is summarized by various experiments that novel chemical formulations could be an important component of integrated disease management against globally threatening maize crop fungal rots.

CONCLUSION

Knowledge of the sensitivity of different fungal species to active substances is extremely important during planning of protection systems and to choose the most effective fungicides. Accurate diagnosis of the etiology of crop diseases, as well as the correct identification of *Fusarium* pathogen and determination of its area, are necessary to predict the effect of used

fungicides. In present studyin-vitro efficacy of three systemic fungicides against *Fusarium verticillioides* revealed that mean radial mycelial growth of the test pathogen was strongly inhibited by application of applied systemic fungicides. Summarization of our results signified that trifloxistrobin 25% + tebuconazole 50% precisely controlled the maximum radial growth of *F. verticilloide*. It is therefore suggested that timely use of these fungicides may help in enhancing overall maize yield with good economic returns.

Conflict of Interest

The authors declared no potential conflict of interest.

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