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Isolation, Evaluation and Characterization of Antagonistic Fungal Strains Against *Rhizoctonia Solani* Causing Black Scurf Disease of Potato

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Abstract-

The present research work aims to control black scurf disease by various methodological approaches. Control measures viz. extracts of medicinal plants, chemicals and bio control fungal agents was investigated. The significance of this research work is to analyze and find the effective control measures to reduce the effect of *Rhizoctonia*. Phytochemical extracts from the leaves of medicinal plants control the fungus at 100ppm and 200ppm effectively under *in vitro* conditions. Copper sulphate and Potassium dihydrogen phosphate showed antifungal activity was effectively at 1% and 0.9%. Antagonistic activity of two fungus *Aspergillus niger*and Aspergillus*nidulans*was positive over the *Rhizoctonia solani*.

Key words Black Scurf. *Rhizoctonia solani.* Phytochemical Extract. *Aspergillus niger.* Copper Sulphate

Introduction

Solanum tuberosum is the world's fourth significant nourishment crop after wheat, rice and maize as a result of its extraordinary yield potential and high nutritive worth. It comprises about portion of the world's yearly yield of all root and tuber crops. Potatoes are developed in around 150 nations all through the world and in excess of a billion people overall expend potatoes. About 328.87 million tons of potato are created on the planet over a region of about 19.13 million hectare. Territory under potato development is practically steady and same is the situation with generation. In any case, there is a slight decrease in the yield. The main ten makers on the planet are China, Russia, USA, Ukraine, Poland, Germany, Belarus, Netherland and France (Hijmans and Robert 2001). These nations together contribute about 70% of the complete creation. India positions third contributing around 7.5% of the world's creation. Europe is the biggest per capita customer, trailed by North America. India rank

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fourth in region and it is the third biggest nation on the planet underway of potato after China and Russian league.Potatoes are used at large scale in many industries and processed into starch, alcohols, glucose, lactic acid etc. Fresh potatoes are an important source of vitamin B & C. Potato is one of the important crops, which have major pathological problems. As potato is one of the famous edible vegetable cropsof the world, many attempts are being made to improve the quality and increase the production of potatoes. There are factors viz. temperature, environmental factors that are responsible for the damage of the potato crop, but the fungal diseases cause the main destruction. Disease such as late blight, early blight affects the crop foliage whereas black scurf disfigures the tubers and reduces the market value and yield.

Black scurf of potato was reported in Maine in 1913 by Morse and Shapovalo in the Maine Agricultural station Bulletin,' The*Rhizoctonia* Disease of Potatoes''. Black scurf is caused by *Rhizoctoni*a *solani and* also called *Rhizoctonia* canker. It is one of the most seasoned and most basic contaminations of potato stems and stolons beneath the dirt surface and result in stem ulcer though sclerotia created by the pathogen on tuber are alluded to as dark scurf (Kotcon et al.1985).*Rhizoctonia* contamination can happen on potato plants whenever from planting to collect. Development of potato plants tainted by *R.solani* is postponed in all stages(Hide et al.1985).Severity of stem canker depend on the initial inoculums concentrations where as severity of black scurf is determined by the inoculums present at the end of the growing season (Scholte, 1992).Improvement of the sclerotia is identified with tuber development and the strength of the root and stolon framework. Rhizoctonia colonizes the subterranean potato plant surface in light of root and shoot exudates (Jeger et al. 1996).It multiplies on the root/stolons framework to shape a broad system of anastomosing hyphae. During the colonizing stage, the host plant remains manifestations less as long contamination structure are not shaped. The progression of contamination is started by progressive spreading of sprinter hyphae bringing about the development of short swollen cells offering ascend to disease pads (Keijer, 1996).Infection cushions are believed to be prerequisite to induce stem and lesions (Keijer et al.1997).*Rhizoctonia solani*, the most broadly perceived types of *Rhizoctonia* was initially depicted by Julius Kuhn on Potato in 1858. *Rhizoctonia solani,* is a basidiomycete growth that doesn't deliver any agamic spore called conidia and just every so often will the organism produce sexual spores called basidiospores.*R. solani* reproduces asexually and exists primarily as vegetative mycelium in nature. The basidiospores are not enclosed in a fleshy, fruiting body as usually occur in

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many basidiomycete fungi. The sexual phase of *R.solani* is known as *Thanatephorus* cucumber is the ideal phase of this pathogen, *Thanatephoruscucumeris*, happens on stem simply over the dirt line as a whitish dark tangle on which basidiospores are shaped, giving the surface a fine appearance. On the tuber surface Rhizoctonia structures sclerotia, dark structure 1-10mm in breadth. Dark to dull darker sclerotia are the resting phase of the parasite on the outside of the develop tubers (Carling and Leiner,1986).Fungus is present on the most tuber seeds, and this is the main means of introducing the disease into new areas (Wicks et al.1996).Various methods have been used to prevent the disease caused by fungus with the help of various biological and chemical methods. Chemical control may be defined as the application of pesticides to control pests, using herbicides to kill tree (Frank, 1981).Chemical control has been used to control post harvest diseases of fruit like apple, orange and lemon fruits. The chemical used to control the pathogen which considered being pathogenic to these fruits were bavistin, benlate, dithane M-45 and sodium bicarbonates. These chemicals proved very much effective when the fruit were treated with these chemicals before the infection occur(Rahamahet al.2000).Certain aldehydes which are plant volatile like acetaldehyde, benzaldehyde and cinnamaldehyde have been used earlier to control the *Rhizopus* rot disease of Apricot (Abdet al. 2008). Many fungicides can be used to control the turf grass diseases caused by fungus such as azoxystrobin, boscalid, captan, chloroneb (Vincelli and Powell, 2009).As chemical causes the development of the resistant strains of the pathogen have resulted into the use of biological controls strategies. Biological control may be defined as the control of pathogenic organisms by various usually nonchemical means, as by the use of biological strains and natural products. The use of the extract of Jordanian medicinal plants viz.*Crupinacrupinastrum, Teucrium polium and Achillea santolina*shows the inhibitory effect against plant pathogen fungi(Dababneh and Khalil,2007).Aqueous extract of certain plants like C*ymbopogan citrates*(Lemongrass), *Eucalyptus camaldulensis*(Eucalyptus) and *Azadirachtaindica*(Neem) were screened for inhibitory activity against *Colletotrichum graminicola,Phomasorghina and Fusarium moniliform*ein naturally infected sorghum seeds (Somdaet al.2007).*Aspergillus niger* and *Aspergillus nidulans*sshows the best antagonistic activity against*Rhizoctonia solani*. The aim of present research work is to control black scurf biological approaches.

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Materials and Methods

Sample collection- -In order to isolate the pathogenic fungus the disease infected tubers were collected from agricultural fields of Gurdaspur district of Punjab. Infected tubers were sealed in the polybagand placed in freezer at 2^0C.

Isolation of pathogenic fungus from diseased tubers-Diseased tubers collected from the fields were washed properly with distilled water to remove soil and kept it in a sterilized Petri plate. Isolation of pathogenic fungi is done as per the method of (Waksman and Fred 1922) with minor modification. In this method, agar medium was poured in a sterile Petri dish and allow the medium to solidify. Now with the help of sterilized scalpel to remove sclerotia from infected tubers and inoculate it on to the solidified PDA medium. Inoculated plates were then incubated at 28^0Cfor next few days and any mycelia growth observed over the medium was purified by transferring the mycelia growth on to fresh PDA medium.

Confirmation of Pathogenic Fungus-In order to establish that the isolated fungi from the diseased tuber is the pathogenic fungus responsible for causing disease, some experiments were designed to establish the involvement of isolated fungi with black scurf disease of potatoes. To confirm the above postulate the following experiment was designed. 1 kg of soil was sterilized at 160^0C for 3-4 hours in oven. After dry heat sterilization it was autoclaved at 15 psi for 15 min. 200 gm sterilized soil is filled in polybag uniformly. Few healthy potatoes are placed on the surface of soil and along with it five blocks of actively growing fungus was placed. Now cover the second layer sterilized soil uniformly. Polybag along with healthy potato tubers and fungus was placed under environmental conditions at 25^0C. Observation was recorded at regular intervals every 2^{nd} day up to 16^{th} day.

Identification of Pathogenic Fungus- Lactophenol cotton blue stain is used for identification under microscope andidentified according to Gilman, 1995.

Preparation of Plant Extract- Two medicinal plants viz. *Achyranthes asper a*and*Eucalyptus* sp. were taken to determine their antifungal properties. Fresh leaves of both medicinal plants were used for preparation of plant extract. Methanolic extract and the antifungal activity is performed as per the method described by Oket al,2008. Ground sample (leaves) (250gm) was extracted three times with 500ml methanol. The extract was filtered and the filtrate was evaporated at a temperature not exceeding 55^0C.The concentrate were resolved with 30 ml methanol for the measurement of the antifungal activity. Antifungal activity of extract was

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evaluated at various concentrations ranging from (100-1000 ppm) by using the agar well method (Ok et al. 2008).

Preparation of Chemicals

Two chemicals viz. Copper Sulphate ($CuSO_4$) and Potassium dihydrogen phosphate (KH_2PO_4) were taken to evaluate the antifungal activities for controlling black scurf disease. Chemicals of different concentrations ranging from 0.1%-1% were prepared as per the method described by (Vyas, 2005). 1gm of test chemical is dissolved in 100ml of water for preparing 1% concentration solution. 0. 9% concentration has been formed by adding 0.9 gm of chemical in 100ml of water. Concentration ranging from 0.8%to 0.1% is prepared in the same way. Antifungal activity was evaluated according to the Agar Well Method as method described by (Ok et al.2008).

Agar Well Method- Antifungal activity of phytochemical extract was evaluated by Agar Well Method (OkOhet al.2008) .Single well (5mm) was prepared at the centre of the Potato Dextrose Agar plate with the help of cork borer of size 5mm diameter. A sterile filter paper disc was placed on the bottom of the well and well was filled with extract of various concentrations of phytochemical extract. Allow the extract to completely evaporate for 1hr.Agar plug (2mm) cut from the actively growing culture (5 day old) of *Rhizoctonia solani* was placed on the surrounding of the well exactly 2 cm away from well. Inhibition of fungal growth was examined by measuring the diameter of inhibition zone and growth of fungi mycelium mat (in mm) on the plate was recorded after 24, 48 and 72hrs respectively. Incubation temperature for the experiment is 28^0C.

Antagonistic activity of Bio control fungal Strains. Antagonistic activity of *Aspergillus nidulans*, *Aspergillus niger* was evaluated according to the method described by Anejas(2008). Pour the Petri plates with PDA medium and allow it to solidify. Inoculate the pathogenic and test fungus at the distance of 1cm apart. Antagonistic activity was evaluated by measuring the growth of colony (in mm) of the both fungus.

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RESULTS

Isolation of Pathogenic Fungus-After 48 hours of incubation, white coloured cottony growth appeared on the surface ofPDA plates. These colonies were further purified on the freshly poured PDA medium in the petri plates with the help of inoculation needle at 25^0C for further growth. White coloured colonies obtained on the surface of Petri plates after 96 hr of incubation (Fig.1). Pure culture of *R.solani* was preserved by making slants of PDA at 2^0C for further experimentation.

Fig.1:Growth of *Rhizoctonia solani* on PDA plate after 5th day of incubation

Confirmation of the fungus- No symptoms was observed on 6th day of incubation of isolated fungus on healthy potato tubers. On 8th day of incubation minute sized brownish dots were observed and on 10th, 12th, and 14th day of incubation tubers were covered with 3-5 sclerotia. On 16th day of incubation large no. of sclerotia are found spreading completely over the surface of the tuber making the tuber completely uneconomical. Generally it was observed that after 16 days of regular observation symptoms of the disease observed on the surface of the healthy potato similar to that found on the surface of diseased tubers. It confirmed that fungus isolated from the diseased tubers was pathogenic in nature. Sclerotia are superficially irregularly shaped; small visible blotches raised lumps (Wharton et al.2007). Cereseni also reported isolation of *Rhizoctonia solani* on modified Ko and Hora medium for quantitative determination of *R.solani* from soils to estimate the inoculums density on the saprophytic/pathogenic competitive ability of the fungus (Cereseni, 1999).

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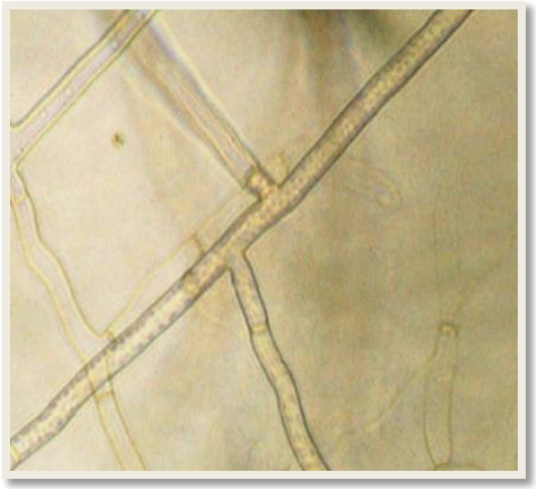
Identification of the Pathogenic Fungus-The pathogenic fungus was identified under microscope according to the manual of soil fungi by Joseph C Gilman (1995). The colony shows the characteristics features viz. white to buff in colour having thread like growth called hypha. Hyphae bear special type of cross wall.The vegetative mycelium of *R.solani* is colourless when young and become brown in colour as they grow and mature. Microscopic studies reveal that hyphae strongly stainable in aniline blue. Branching at right angles is present often with the formation of cruciform cells (Fig.2). Basidia are sub cylindricalin shape not greatly exceeding in diameter of supporting cells, relatively short, bearing four or six to eight sterigmata. Spores are smooth walled or rarely asperulate or spinulose, colorless or pale ochraceous.

Fig.2 Hyphae of *Rhizoctonia solani* under microscope



Antifungal activity of medicinal plants : Antifungal and antimicrobial activity of the medicinal plants have supported the use of medicinal plant as antifungal agent (Akinpelu and Onakoya, 2006).In the present research work two medicinal plants i.e*Achyranthes aspera*and *Eucalyptus*sp was used.

Antifungal Activity of *Achyranthes aspera:* Antifungal activity were observed at various concentration ranging from 100 -1000 ppm. At 100ppm, after 24hrs of incubation inhibitory zone (IZ) of 10mm was observed with Colony Size (CS) of 3 mm was observed. After 48hr of incubation, IZ and CS was formed to be 8mm and 5mm respectively and with further increase in the incubation period resulted into IZ and CS of 6mm and 7mm respectively. At 200 ppm,results were completely similar to the result obtained at 100ppm.At 300ppm, IZ and

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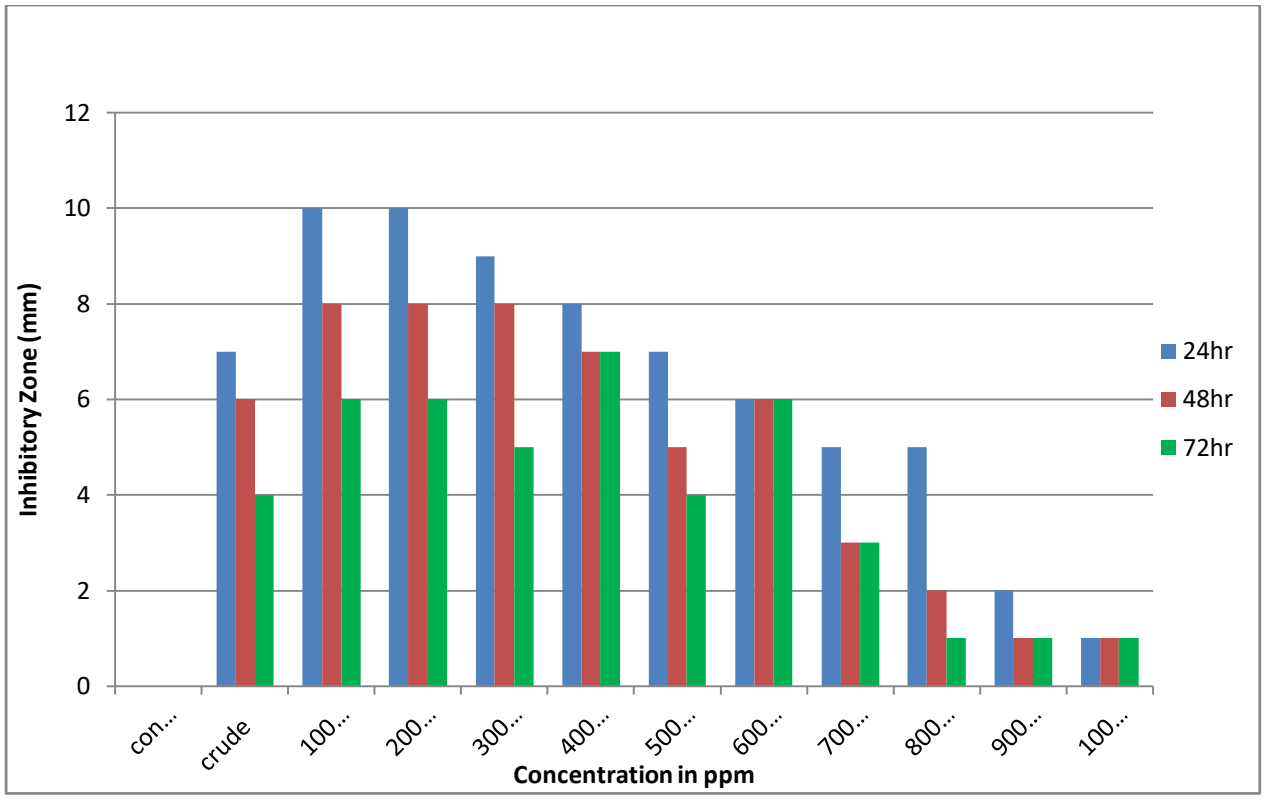
CS were found to be at somewhat different with 9mm and 5mm (24hr), 8mm and 6mm (48hr) and 5mm and 7mm (72hr) respectively. With the decrease in concentration further at 400, 500, 600, 700, 800, 900 and 1000ppm the colony size growth is tremendousand with negligible inhibitory zone. At maximum dilution i.e. at 1000ppm, IZ and CS were 1mm and 18mm (24hr), at all the three incubation periods. The results when compared to control consisting of filter paper soaked in distilled water resulting into IZ and CS of 0mm and 15mm (24hrs), 0mm and 17mm (48hr), and 0mm and 18mm (72hr) respectively. Fig.3 depicts the antifungal activity of *Achyranthesaspera* against the *Rhizoctonia solani*. It is evident from the result that *Achyranthesaspera* shows significant amount of antifungal activity.

Fig-3:EFFECT OF LEAVES EXTRACT OF *Achyranthes aspera AT* VARIOUS CONCENTRATIONS ON THE GROWTH OF*Rhizoctonia solani*



The results indicate that leaves of *Achyranthes aspera at* 100ppm and 200ppm is found to be having significant antifungal activity which could be used for controlling *Rhizoctonia solani* fungus under *in vitro*conditions.

Antifungal activity *of Eucalyptus sp.:* *Eucalyptus sp.* has shown antifungal activity against the *Rhizoctonia solani*(Fig.4).It is evident from the results that *Eucalyptus* shows significant amount of antifungal activity. Antifungal activity was observed at various

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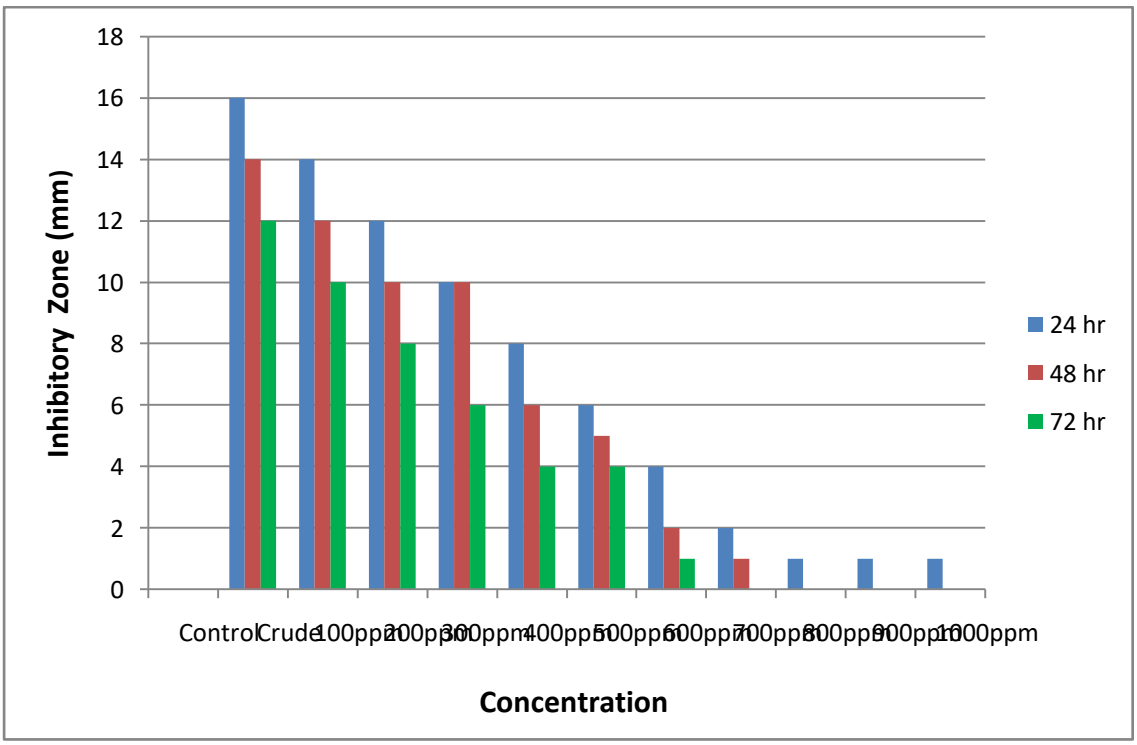
concentration ranging from 100 -1000 ppm. At 100ppm, after 24hrs of incubation inhibitory zone (IZ) of 14 mm was observed with Colony Size (CS) of 1 mm was observed. After 48hr of incubation, IZ and CS was formed to be 12 mm and 2 mm respectively and with further increase in the incubation period resulted into IZ and CS of 10 mm and 3 mm respectively. At 200 ppm, IZ and CS are formed of 12 mm and 2mm after 24hr of incubation, 10 and 4 mm after 48hr and 8 and 5mm after 72hr respectively. At 300ppm, IZ and CS were found to be at somewhat different with 10mm and 3mm (24hr), 10 mm and 4mm (48hr) and 6mm and 8 mm (72hr) respectively. With the decrease in concentration further at 400, 500, 600, 700, 800, 900 and 1000ppm the colony size growth is tremendousand with negligible inhibitory zone. At maximum dilution i.e. at 1000 ppm, IZ and CS were 1 mm and 16 mm (24hr), 0 mm and 18 mm at other two incubation periods. The results when compared to Control consisting of filter paper soaked in distilled water resulting into IZ and CS of 0 mm and 15 mm (24hrs), 0mm and 16 mm (48hr), and 0mm and 19 mm (72hr) respectively. The results indicate that extract of the leaves of *Eucalyptus sp.*at 100ppm and 200ppm is found to be having significant antifungal activity which could be used for controlling *Rhizoctonia* fungus under *in vitro* conditions (Fig.4).

Fig 4: EFFECT *OF LEAVES EXTRACT OF Eucalyptus* sp. AT VARIOUS CONCENTRATIONS ON THE GROWTH OF *Rhizoctonia solani*



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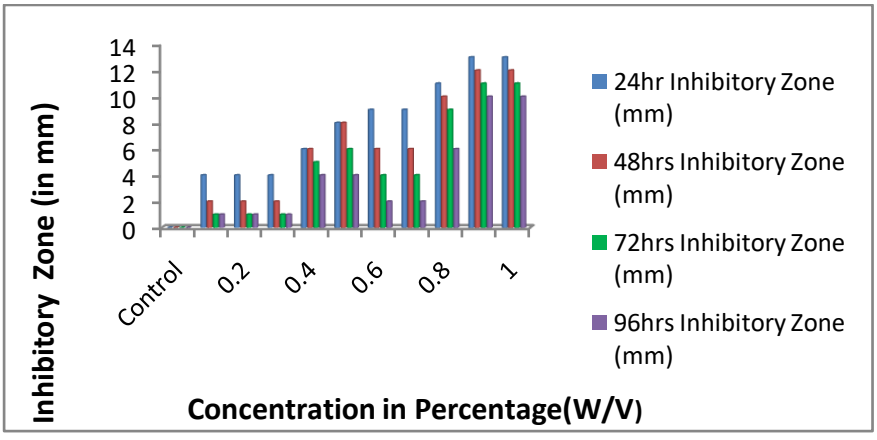
Antifungal activity of chemicals: Two chemicals viz. Copper sulphate and Potassium Dihydrogen Phosphate were used in the present work to check its antifungal activity against the pathogenic fungus *Rhizoctonia solani.*

Antifungal Activity of Copper Sulphate ($CuSO_4$): Antifungal activity of Copper Sulphate ($CuSO_4$)was evaluated at various concentrations ranges from 0.1% to 1% (W/V)(Fig.5).Inhibitory Zone (IZ) of 13mm was observed with Colony Size (CS) of 3mm at 1% after 24hr of incubation.After 48hr of incubation, IZ and CS were found to be 12mm and 4mm. With further increase in incubation periodi.e 72 and 96 hrs resulted into IZ and CS of 11 mm and 5 mm, 10 and 6mm respectively. At 0.9%, results obtained were completely similar to the 1%. At 0.8%, IZ and CS are foundto be 11 and 4mm (24 hr), 10 and 5mm (48hr), 9 and 6 mm(72hr) and 6 and 8 mm (96hr). With further decrease in the concentration of salt at 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, theCS increased and IZdecreased. At less concentration of the salt, the IZ and CS are of 4 and 10 mm (24hr), 2 and 12 mm (48hr), 1 and 14 mm (72hr) and same at 96hr of incubation.It is clearly evident from the size of the inhibitory zones (IZ) and growth of the Colony size (CS) that $CuSO_4$ shows strong antifungal activity at 1% and 0.9% concentrations strongly. At these concentrations fungus shows inability to grow. The results when compare with control which consist of filter paper disc soaked in distilled water resulting into IZ and CS of 0 and 14 mm (24hr), 0 and 16 mm (48hr), 0 and 17 mm(72hr), 0 and 18(96 hr) respectively.

Fig 5: EFFECT OF Copper Sulphate AT VARIOUS CONCENTRATIONS ON GROWTH OF*Rhizoctonia solani*



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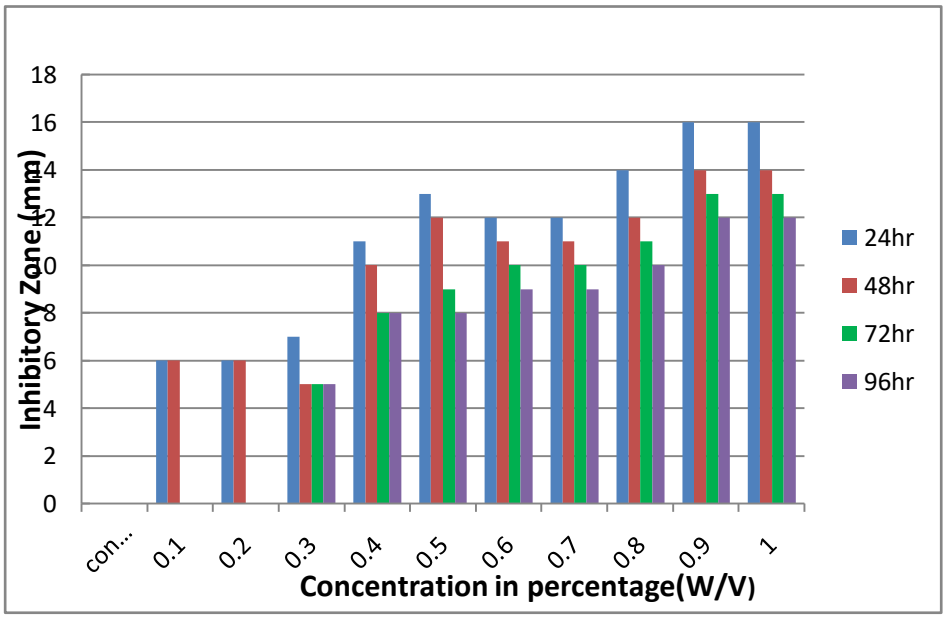
Antifungal Activity of the Potassium Dihydrogen Phosphate (KH_2PO_4)

Antifungal activity of Potassium Dihydrogen Phosphate (KH_2PO_4) was evaluated at various concentrations ranges from 0.1% to 1% (W/V) (Fig.6).1% IZ of 16 mm was observed with CS of 4 mm at 24 hr of incubation.After 48hr of incubation, IZ and CS were found to be 14mm and 6 mm. With further increase in incubation period resulted into IZ and CS of 13 mm and 7mm (72hr), 12 and 8mm (96hr) respectively. At 0.9%, results obtained were completely similar to the 1%. At 0.8% IZ and CS are formed of 14 and 5mm (24 hr), 12and 7 mm (48hr), 11 and 8mm (72hr) and 10 and 9mm (96hr). With further decrease in the concentration of salt at 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, the CS increase and IZ decreases. At less concentration of the salt at 0.1%, the IZ and CS are of 6 and 14 mm (24hr), 6 and 14 mm (48hr), 0 and 16 mm (72hr) and same at 96hr of incubation. $KH_2PO_4$was also found to be showing strong antifungal activity. It inhibits the growth of the fungus colony at 1% and 0.9% concentrations strongly. At these concentrations fungus shows inability to grow. The results when compare with control which consist of filter paper disc soaked in distilled water resulting into IZ and CS of 0and 18 mm(24hr),0 and19 mm(48hr),0 and 19 mm (72hr) and same after 96 hr of incubation

Fig. 6 Effect of Potassium dihydrogen phosphate at various concentrations on the Growth of *Rhizoctonia solani*



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ANTAGONISTIC ACTIVITY OF BIOCONTROL FUNGAL STRAINS

Antagonistic Activity of *Aspergillus nidulans*:

*A.nidulans*has shown the antagonistic activity against the *Rhizoctonia solani* (Fig. 7).The data reveals that *A.nidulans*inhibits the growth of the *Rhizoctonia solani*. After 24hr of incubation, the Colony size (CS) of *A.nidulans* and *Rhizoctoniasolani*was found to be 2 and 0 mm. CS was found to be 70 mm and 1mm (48 hr), 90 mm and 2 mm (72 hr) respectively. The results after 96hr and 120 hr of incubation were completely similar to that of the 72hr. When the results were compared with the control of the *A.nidulans*, it was found that the CS was 10mm (24hr), 70mm (48hr), 90mm (72hr), 110mm (96hr) and 130mm (120hr) respectively.Control of the*Rhizoctonia* shows the growth of Colony 10mm (24hr), 15mm (48hr), 18mm (72hr),20 mm after 96hr and 120hr of incubation respectively.

Antagonistic Activity of the *Aspergillus niger*-

A.niger has shown the antagonism against *Rhizoctonia solani.*The result clearly indicates that *A.niger* inhibits the growth of the *Rhizoctonia solani*and shows strong antagonistic activity against *Rhizoctonia*sp when growing under close vicinity. After 24hr of incubation,CS of *A.niger* and *Rhizoctonia* was 2 mm and 1 mm respectively.CS was found to be 15mmand 2 mm (48 hr), 40mmand 2 mm(72 hr) respectively. The results obtained after 96hr were similar to that of the 72hr.When the results were compared with the control of the *A.niger*growing independently, it was found that the CS was 2mm (24hr),60mm (48hr), 80mm (72hr),100mm (96hr) respectively.Control of the*Rhizoctonia*sp shows the growth of colony 10mm (24hr), 15mm(48hr), 18mm(72hr) and 20mm (96hr) respectively(Fig.8)

Fig. 7: ANTAGONISTIC ACTIVITY OF *A.nidulans* AGAINST *Rhizoctonia solani*

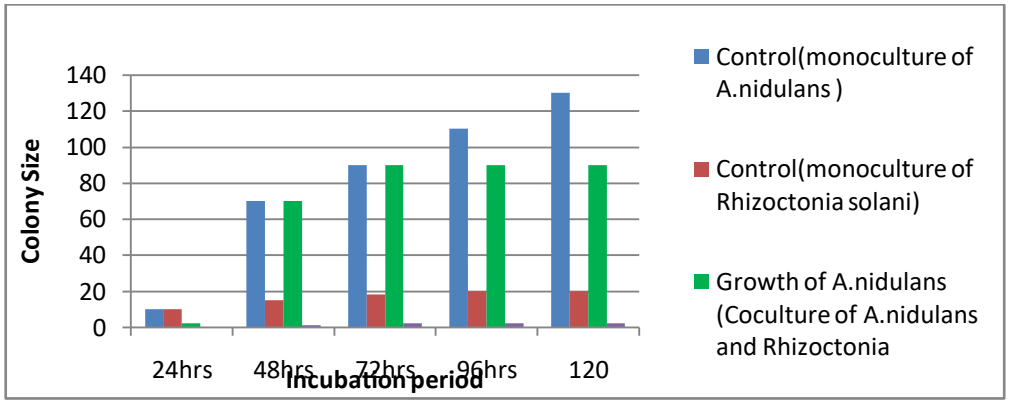

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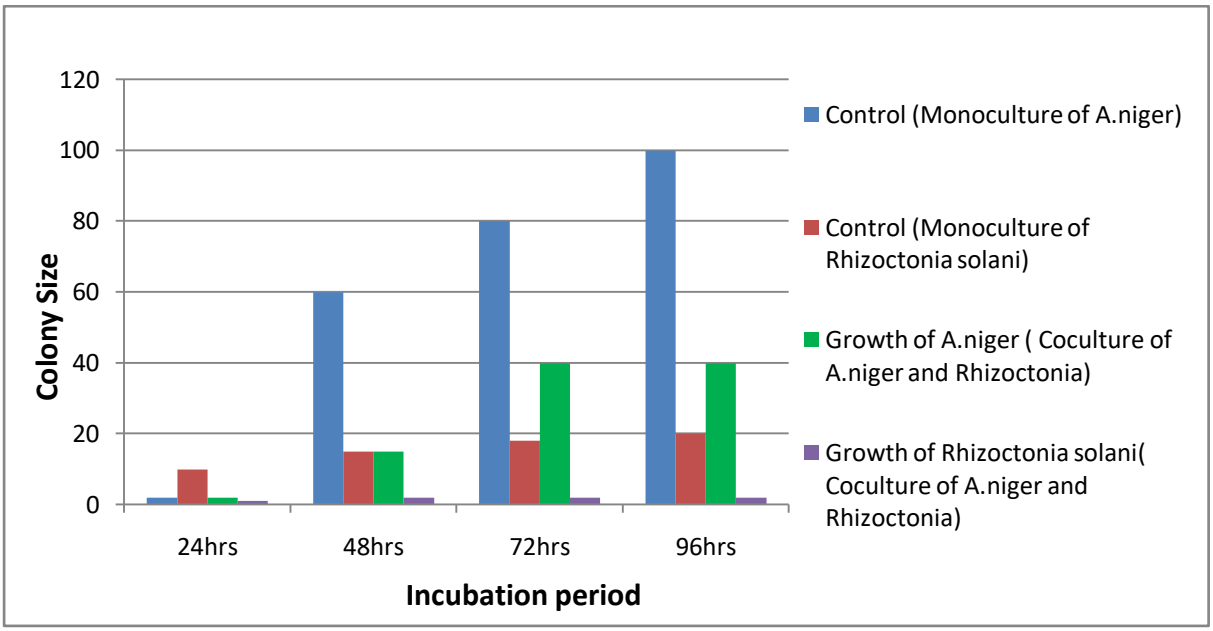
Fig. 8:ANTAGONISTIC ACTIVITY OF *A.niger* AGAINST *Rhizoctonia solani.*



Discussion: Black scurf disease of Potato caused by *Rhizoctonia solani* is one of the major fungal diseases of potato which disfigures the tubers and reduces their yield and economicvalue. The management of this disease is difficult by single control measures because of soil borne nature and very high degree of survival of this pathogen. In the present investigation, attempt has been to control measures viz. efficiency of some medicinal plants, chemicals and biocontrol fungal agentswere investigated. Two medicinal plants namely *Achyranthes aspera* and *Eucalyptus* sp. were evaluated *in vitro* against the fungus for their antifungal activity. Leaves extract of medicinal plants control the fungus at 100ppm and 200ppm effectively under *in vitro* conditions. Research finding establish the potentiality/efficacy of *Achyranthes aspera* and *Eucalyptus* sp. to be used for controlling black scurf disease. Antifungal activity of Copper Sulphate ($CuSO_4$) and Potassium dihydrogen Phosphate (KH_2PO_4) were used. Chemicals were prepared at various concentrations ranging 0.1 to 1% (W/V). The chemical were applied against the fungus isolated from the diseased tubers under *in vitro* conditions by using agar well method. These chemicals control the growth of pathogenic fungus effectively at 1% and 0.9%. Antagonistic activity of two biocontrol fungus namely *Aspergillus nidulans* and *Aspergillus niger* was evaluated specifically against *Rhizoctonia solani*. In the present research work efforts have been done to control the growth of the *Rhizoctonia solani*by the *A.nidulans*and*A.niger*on the

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surface of PDB medium by measuring the growth of the colony of both funguses at different hours of incubation with *Rhizoctonia solani*. Both fungus *A.nidulans* and *A.niger* show tremendous growth over the *Rhizoctonia solani* and prevent it to grow.

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